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INSERTION OF DOUBLE FURIN CLEAVAGE SITES IN SENDAI VIRUS FUSION PROTEIN MIMICS THE SYNCYTIAL AND ATTACHMENT PROTEIN-INDEPENDENT PROPERTIES OF RESPIRATORY SYNCYTIAL VIRUS

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At every moment of our lives we all have one foot in a fairy tale and the other in the abyss.
Paulo Coelho

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"On even the worst days, when nothing was working at the lab, I knew that at home I would find warmth, peace, companionship, and encouragement. As a consequence, the next day would surely be better".
James Cronin (Nobel Prize in Physics 1980)

Vielen Dank für deine tatkräftige Unterstützung in den vergangenen Jahren. Eigentlich, solltest du auch Doktor werden!

TABLE OF CONTENTS

ABBREVIATIONS

Amp^R	Ampicillin resistance
AEC	3-amino-9-ethylcarbazole
AMEM	Alpha Minimum Essential Medium
ATCC	American type culture collection
BHK	Bovine hamster kidney cells
bp	Base pairs
BRSV	Bovine respiratory syncytial virus
BSA	Bovine serum albumin
BSR/T7-5	BHK-21 cells that constitutively express T7 polymerase
cDNA	Complementary deoxyribonucleic acid
cRNA	Complementary ribonucleic acid
CDV	Canine distemper virus
CHO	Chinese hamster ovary cells
CT	Cytoplasmic tail
DMEM	Dulbecco’s Modified Eagle Medium
DMSO	Dimethyl sulphoxide
ECL	Enhanced chemiluminescence
ER	Endoplasmic reticulum
ECMV	Encephalomyocarditis virus
EDTA	Ethylenediaminetetraacetic acid
F	Fusion protein
FCS	Foetal calf serum
Ffu	Focus forming units
FI-RSV	Formalin-inactivated respiratory syncytial virus
FITC	Fluorescein isothiocyanate
FP	Fusion peptide
F_RSV	Respiratory syncytial virus fusion protein
F_SeV	Sendai virus fusion protein
G	Glycoprotein
GAGs	Glycosaminoglycans
GE	Gene end signal

GS	Gene start signal
Gm	Membrane glycoprotein
Gs	Soluble glycoprotein
h	Hours
H(A)	Haemagglutinin
HBD	Heparin binding domain
HEPES	4-(2-hydroxyethyl)-1-piperazineethane-sulphonic acid
HIV-1	Human immunodeficiency virus type 1
HMPV	Human metapneumovirus
HN	Haemagglutinin-neuraminidase
HPIV	Human parainfluenza virus
HRA/B	Heptad repeat A/B
HT	Hypoxanthine-thymidine
Ig	Immunoglobulin
i.m	Intramuscular
i.p	Intraperitoneal
kB	Kilobases
L	Large polymerase
LB	Lysogeny broth
Lec2	Sialic acid-deficient Chinese hamster ovary cells
LLC-MK2	Rhesus monkey kidney cells
Luc	Luciferase
M	Matrix protein
MAb	Monoclonal antibody
min	Minutes
MCS	Multiple cloning site
MOI	Multiplicity of infection
mRNA	Messenger ribonucleic acid
MW	Molecular weight
N	Nucleoprotein
NA	Neuraminidase
NDV	Newcastle disease virus
NS1/NS2	Non-structural proteins
ORF	Open reading frame

OPD	o-phenylene diamine
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pep27	27 amino acid peptide
PIV5	Parainfluenza virus 5
P	Phosphoprotein
PO	Peroxidase
Pro-5	Chinese hamster ovary proline auxotroph cells
rSeV	Recombinant Sendai virus
RNP	Ribonucleoprotein complex
RPE	R-phycoerythrin
rpm	Revolutions per minute
RT	Room temperature
RT-PCR	Reverse transcriptase polymerase chain reaction
SARS	Severe acute respiratory syndrome
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
sec	Seconds
SH	Small hydrophobic protein
SP	Signal Peptide
TEMED	N-N-N’-N’-tetramethyl ethylenediamine
TM	Transmembrane region
TNF	Tumour necrosis factor
UTR	Untranslated region
vol	volume
vRNA	Viral ribonucleic acid
vRNAP	Viral RNA-dependent RNA polymerase
wt	Wild-type
6HB	6 Helix bundle

ABSTRACT

Cell entry by paramyxoviruses requires fusion between viral and cellular membranes. Paramyxovirus infection also gives rise to the formation of multinuclear, fused cells (syncytia). Both types of fusion are mediated by the viral fusion (F) protein, which requires proteolytic processing at a basic cleavage site in order to be active for fusion. In common with most paramyxoviruses, fusion mediated by Sendai virus F protein (F_SeV) requires co-expression of the homologous attachment (HN, haemagglutinin-neuraminidase) protein, which binds to cell surface sialic acid receptors. In contrast, respiratory syncytial virus fusion protein (F_RSV) is capable of fusing membranes in the absence of the viral attachment (G) glycoprotein. Moreover, F_RSV is unique among paramyxovirus fusion proteins since F_RSV possesses two multibasic cleavage sites, which are separated by an intervening region of 27 amino acids. We constructed a series of chimeric F_SeV mutants by insertion of one or both F_RSV cleavage sites and various regions of pep27 into F_SeV. Insertion of both F_RSV cleavage sites in F_SeV led to increased cell-cell fusion of transfected cells and a reduced dependency on the HN attachment protein for syncytia formation. In light of the results in transfected cells, we rescued recombinant Sendai viruses (rSeV) expressing mutant F proteins containing one or both F_RSV cleavage sites. All cleavage site mutant rSeV displayed reduced thermostability, with double cleavage site mutants exhibiting a hyperfusogenic phenotype in infected cells. Furthermore, double cleavage site mutant rSeV were less dependent on the interaction of HN with sialic acid for infection, thus mimicking the unique ability of RSV to fuse and infect cells in the absence of a separate attachment protein. Therefore, these results support the hypothesis that the presence of two multibasic cleavage sites represents a strategy to regulate activation of a paramyxovirus F protein for cell-cell fusion in the absence of an attachment protein.

RESUMEN

Los paramixovirus requieren para su entrada en la célula diana la fusión entre las membranas de la célula y el virus. La infección por paramixovirus también produce la fusión de células entre sí, dando lugar a células grandes multinucleadas (sincitios). Ambos tipos de fusión son mediados por la proteína viral de fusión (F), que para su activación requiere que se produzca un procesamiento proteolítico en un sitio de corte de carácter básico. Al igual que la mayoría de los paramixovirus, la fusión mediada por la proteína de fusión del virus Sendai (F_SeV) requiere coexpresión de la proteína de unión al receptor (HN), que se une a residuos de ácido siálico en la superficie celular. Por el contrario, la proteína de fusión del virus respiratorio sincitial (F_RSV) puede producir fusión de membranas en ausencia de la glicoproteína viral de unión al receptor (G). La proteína de fusión F_RSV es única entre los paramixovirus, ya que posee dos sitios de corte multibásicos, separados por una región de 27 aminoácidos (pep27). En este trabajo se han construido una serie de mutantes quiméricos de F_SeV en los que se han insertado uno o los dos sitios de corte de F_RSV y diversas regiones del pep27. La inserción de los dos sitios de corte de F_RSV en F_SeV promovió un aumento de la fusión de células transfectadas y una reducción en la dependencia de la proteína de unión HN para la formación de sincitios. A la vista de los resultados obtenidos en células transfectadas, se procedió al rescate de virus Sendai recombinantes (rSeV), que expresan proteínas F mutantes que tienen uno o los dos sitios de corte de F_RSV. Todos los rSeV con sitios de corte mutados tuvieron una estabilidad térmica reducida, y los mutantes con el doble sitio de procesamiento mostraron un fenotipo hiperfusogénico en células infectadas. Además, los mutantes rSeV con dos sitios de corte mostraron menor dependencia de la interacción de HN con ácido siálico para la infección, imitando así la capacidad específica de RSV para fusionar e infectar células en ausencia de la proteína de unión al receptor. Por lo tanto, estos resultados sugieren que la presencia de dos sitios de corte mutlibásicos representa una estrategia para regular la activación de una proteína de fusión de paramixovirus en ausencia de la proteína de unión al receptor.

1. INTRODUCTION

1. INTRODUCTION

1.1 Classification of paramyxoviruses

The *Paramyxoviridae* family of enveloped, negative-strand RNA viruses (*Mononegavirales)* includes highly prevalent human viruses (measles, respiratory syncytial virus), economically important animal viruses (Newcastle disease virus, rinderpest virus), and the recently identified Hendra and Nipah viruses that cause fatal disease. Paramyxoviruses are subdivided into two subfamilies: the *Paramyxovirinae* and the *Pneumovirinae* (Collins and Crowe 2007). *Paramyxovirinae* and *Pneumovirinae* can be distinguished morphologically by differences in the size and shape of the nucleocapsids (Bhella *et al*., 2002), and by major differences both in the number of encoded proteins and the nature of the attachment protein (Fig. 1.1).

Sendai virus (SeV) and human respiratory syncytial virus (RSV) are representative members of the *Paramyxovirinae* and *Pneumovirinae* subfamilies, respectively. RSV was first isolated in 1956 from a laboratory chimpanzee with a common cold-like illness (Blount *et al*., 1956), and was subsequently recovered from two children with respiratory illness. Serological studies confirmed that infection of children was common (Chanock *et al*., 1957), and RSV is now recognised as the leading cause of serious paediatric respiratory tract disease worldwide (Nair *et al*., 2010). In addition, RSV may also cause serious disease in the elderly and the severely immunosuppressed (Falsey *et al*., 2005). While RSV is considered an important pathogen for vaccine development, vaccination of infants with formalin-inactivated RSV (FI-RSV) in the 1960s was poorly protective (Kim *et al*., 1969). Furthermore, FI-RSV vaccination was associated with increased severity of disease on subsequent natural infection with RSV (Openshaw *et al*., 2001). Natural RSV infection does not produce lasting protective immunity, and repeated infections throughout life are common. Therefore, advances in the knowledge of the biology of RSV remain crucial for the future development of licensed vaccines and antiviral agents.

Sendai virus, also known as murine parainfluenza virus type 1, is responsible for a highly transmissible respiratory tract infection in mice, hamsters and rats. Sendai virus is frequently studied as an archetypal representative of the entire *Paramyxoviridae* family in order to elucidate the molecular and biological properties of the paramyxoviruses (Faisca and Desmecht 2007). Scientific interest in SeV has also increased in recent years due to its potential value as a vector for gene transfer (Kinoh and Inoue 2008).

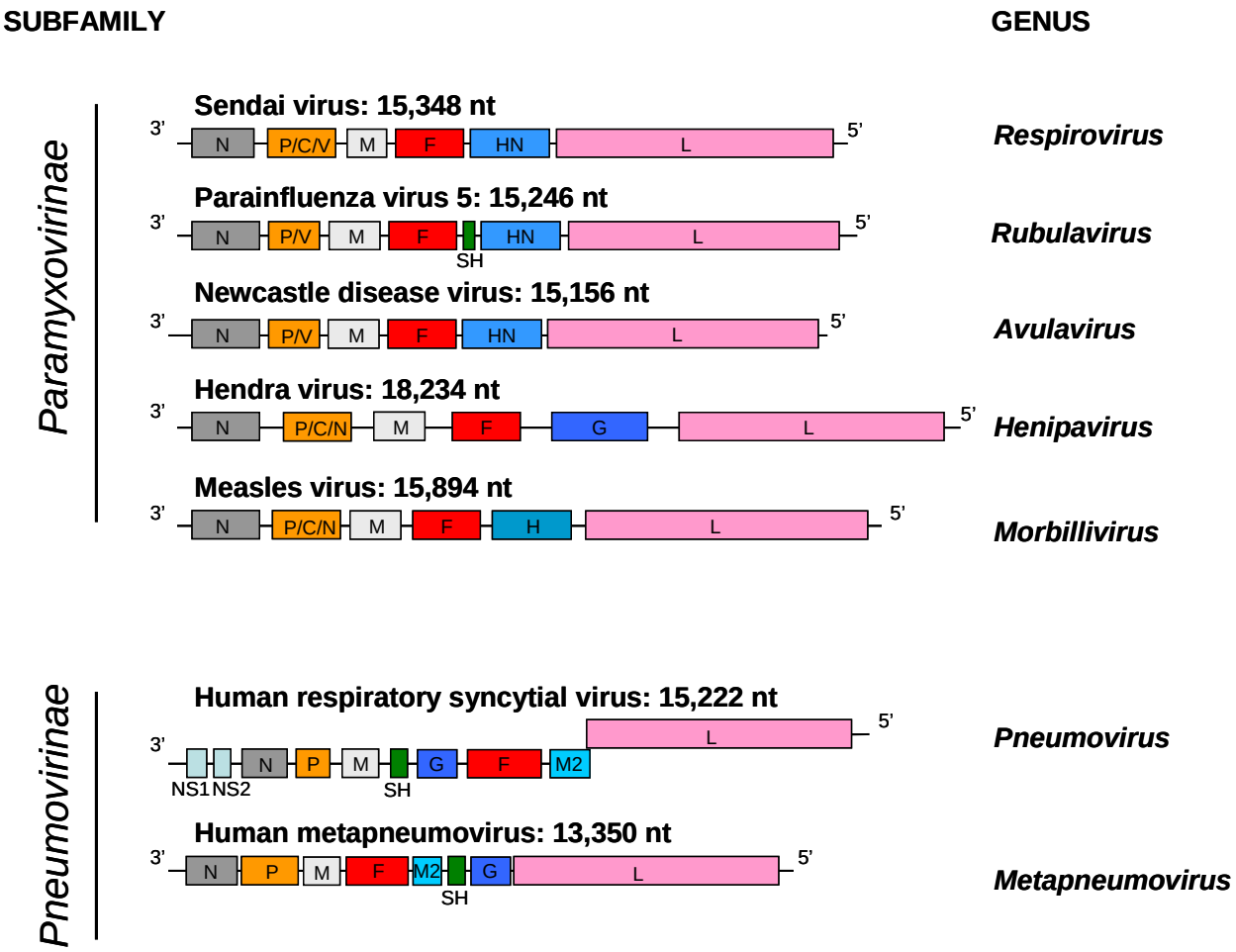


Figure 1.1. Paramyxovirus genome organisation. Gene maps for representative members of each genus of the *Paramyxovirinae* and *Pneumovirinae* subfamilies are shown. The nucleotide length of each viral genome (nt), the intergenic regions and the non-coding termini (not to scale) are shown. Genes are colour-coded for function and the overlap between the RSV M2 and L genes is indicated.

1.2 Molecular Biology of paramyxoviruses

1.2.1 Paramyxovirus genomes and their encoded proteins

Paramyxovirus RNA genomes are non-segmented, single-stranded and 15-19 kB in length. Genomic RNA contains a 3'extracistronic region of approximately 50 nucleotides (leader), and a 5'extracistronic region of 50-161 nucleotides (trailer), which are essential for transcription and replication. Transcription control sequences are also found at the beginning and end of each gene, which are separated by intergenic regions. While respirovirus intergenetic regions are precisely 3 nucleotides, they vary in length for pneumoviruses (1-56 nucleotides). Paramyxovirus genomes typically contain 5-10 tandemly-linked genes (Fig. 1.1), although coding capacity may be increased by the use of overlapping ORFs.

Nucleocapsid proteins N, P, L and M2-1: Paramyxoviruses consist of a stable nucleocapsid core surrounded by a lipid bilayer envelope (Fig. 1.2), which is acquired as the virus buds from the host cell. The nucleoprotein (N) binds to both negative (-) sense genomic and positive (+) sense antigenomic RNA to form the helical nucleocapsid template, which is the biologically active form of viral RNA (vRNA). The crystal structure of an RSV-N protein complex revealed that the N protein is able to form decameric rings that bind to RNA via a basic surface groove (Tawar *et al*., 2009). In addition, N also binds to both the phosphoprotein (P) and the large polymerase (L) to form the ribonucleoprotein complex (RNP), which represents the minimal replication unit for the *Paramyxovirinae* (Grosfeld *et al*., 1995; Yu *et al*., 1995). The L protein is a viral RNA-dependent RNA polymerase (vRNAP), which is responsible for transcription of functional viral mRNA, including 5'capping and methylation and 3'polyadenylation activities (Ogino *et al*., 2005). The L protein is typically found in very low amounts in infected cells. For example, each Sendai virus nucleocapsid is composed of approximately 2600 N, 300 P and 50 L proteins (Lamb *et al*., 1976). While the L protein contains all vRNAP catalytic activities, L binds to the nucleocapsid template via the P protein (Horikami *et al*., 1992). The P protein is phosphorylated on Ser/Thr residues, and is an essential

cofactor for both the L and N proteins. Finally, the M2-1 protein (unique to the pneumovirus genome), is also incorporated into the RSV nucelocapsid. The M2-1 protein is transcribed from the first M2 gene ORF, and acts as an essential transcription factor to promote processivity of the pneumovirus polymerase (Collins *et al*., 1995).

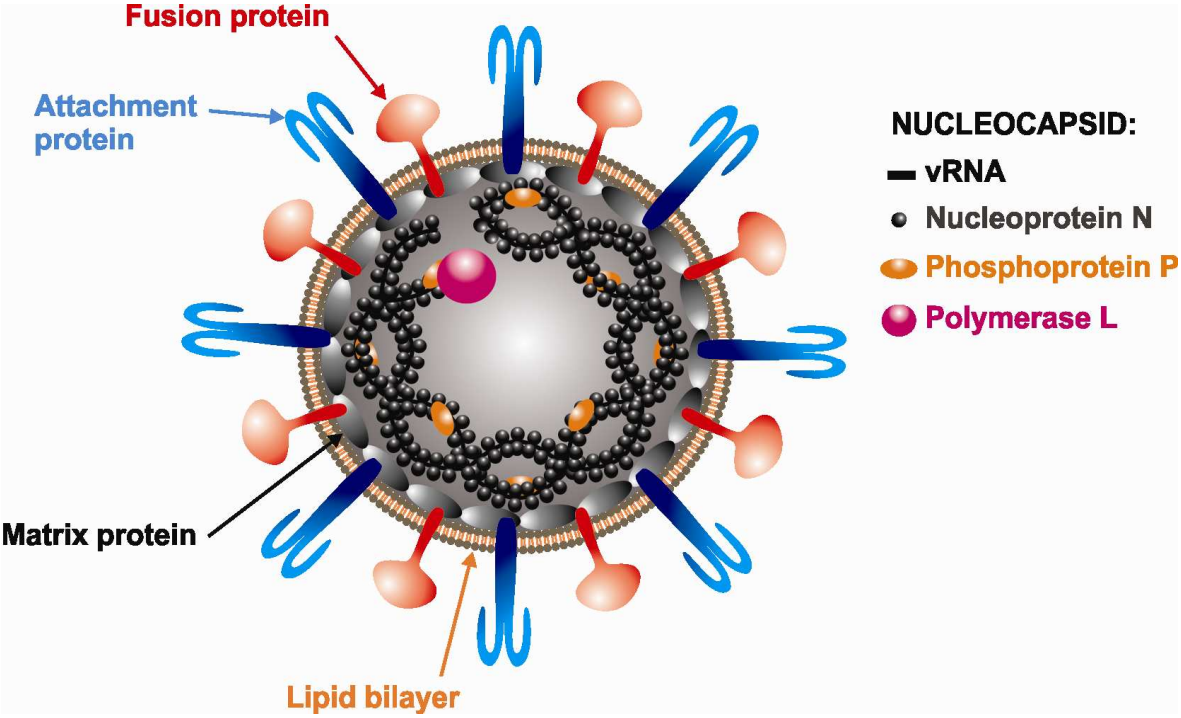


Figure 1.2. The paramyxovirus virion. Schematic representation of a paramyxovirus virion (not drawn to scale), with proteins coloured according to Fig. 1.1. Fusion (F) and attachment (H, HN or G) proteins are inserted into the lipid bilayer envelope. In addition, some paramyxoviruses, including RSV, also contain a small hydrophobic (SH) envelope glycoprotein (not shown). Negative-strand RNA associates with the nucleoprotein (N), phosphoprotein (P), and large polymerase (L) proteins to form the ribonucleocapsid (RNP). Figure provided by Alfonsina Trento (ISCIII, Madrid, Spain).

Matrix protein M: As shown in Fig. 1.2, the matrix (M) protein forms a layer on the inner face of the envelope. The M protein is thought to act as a central organiser of viral morphogenesis via interactions with the cytoplasmic tails of envelope glycoproteins, the lipid bilayer and the nucleocapsid. The ability of the M protein to self associate, combined with its interaction with the nucleocapsid, may provide the driving force for formation of budding virions (for a review, see Ghildyal *et al*., 2006).

Envelope glycoproteins G/H/HN, F and SH: Paramyxoviruses contain two virally encoded integral membrane glycoproteins within the lipid envelope: the fusion (F) and attachment (glycoprotein [G], haemagglutinin [H] or haemagglutinin-neuraminidase [HN]) proteins, which form spikes that extend 8-12 nm from the surface (Fig 1.2). All paramyxoviruses possess a fusion protein that mediates fusion of the viral and cellular membranes during virus entry. However, the nature of the attachment protein, which is responsible for binding of the virus to target cell surface receptors, varies between distinct members of the *Paramyxoviridae*. Paramyxovirus fusion and attachment glycoproteins represent important antigenic targets, since they induce neutralising and protective antibodies (Walsh *et al*., 1987). Given the focus of this thesis on paramyxovirus cell entry, the fusion and attachment proteins will be discussed in detail in section 1.3.

In addition, mumps virus, PIV5 and RSV encode a third integral membrane protein of unknown function: the small hydrophobic (SH) protein. The SH protein is not required for RSV entry or spread *in vitro* (Techaarpornkul *et al*., 2001), although RSV lacking the SH protein is attenuated *in vivo* (Bukreyev *et al*., 1997). Indeed, paramyxovirus SH proteins have been shown to inhibit TNFα signaling and prevent infected cells from undergoing apoptosis, which may be beneficial for virus replication (Fuentes *et al*., 2007; Wilson *et al*., 2006). Furthermore, electron microscopy of RSV SH protein revealed pentameric and hexameric ring-like structures containing a central pore that could allow the passage of ions and small molecules (Carter *et al*., 2010).

Accessory proteins: The paramyxovirus P/V/C gene directs the expression of a number of accessory proteins (V, W, C', C, Y1 and Y2 for SeV) via RNA editing and the use of alternative translation codons. Although non-essential for infectivity, the paramyxovirus accessory proteins exert multiple functions on viral growth, including negative regulation of RNA synthesis and inhibition of cellular components of the type I interferon response (for a review see Goodbourn and Randall 2009). The pneumoviruses are unique within the *Paramxyovirinae* since they do not produce alternative proteins from the P

gene. However, RSV possesses two additional genes that encode non-structural proteins NS1 and NS2, which have been shown to independently inhibit induction of the type I interferon response to viral infection (Spann *et al*., 2004). These non-structural proteins enhance the growth of RSV, but are not essential for virus replication *in vivo* (Teng *et al*., 1999; 2000).

1.2.2 Replication Cycle

The entire replication cycle of the *Paramyxoviridae* takes place in the cytoplasm of the host cell (Fig. 1.3). Viral entry initially involves binding of virions to the target cell surface. For members of the *Rubulavirus* and *Respirovirus* genera, sialic acid-containing molecules serve as cell surface receptors. Sialic acid-containing gangliosides are thought to function as the cellular receptor for the Sendai virus HN attachment protein (Markwell *et al*., 1980; 1984). While the specific cell surface receptor for pneumoviruses is not known, RSV interacts with cell surface glycosaminoglycans, in particular with heparan sulphate (Escribano-Romero *et al*., 2004; Feldman *et al*., 1999; Feldman *et al*., 2000; Hallak *et al*., 2000; Karger *et al*., 2001; Krusat and Streckert 1997; Martínez and Melero 2000; Techaarpornkul *et al*., 2002). Following attachment, the paramyxovirus fusion (F) protein mediates fusion of the viral envelope with the cellular plasma membrane at neutral pH.

Fusion results in the delivery of the nucleocapsid into the cytoplasm, where transcription of the viral genome is carried out by the vRNAP, generating a set of mRNAs that are translated by cellular ribosomes. The vRNAP transcribes the (-) sense genomic template in a sequential manner from a single promoter at the 3’ end of the vRNA to generate capped and polyadenylated mRNAs. Transcription initiates at the first gene start (GS) signal of the 3’-terminal gene and proceeds along the genome until a gene end (GE) sequence is reached. The vRNAP subsequently scans the intergenic region without dissociating from the template and reinitiates at the next downstream GS signal in a “start-stop” mechanism of transcription. This results in a 3’-5’ polar gradient of mRNA abundance, as the likelihood of the polymerase reinitiating at a downstream GS signal is reduced at each gene junction. Upon translation of the primary transcripts and accumulation of viral proteins, the viral genome is replicated to

produce a full-length, (+) sense antigenome (cRNA). The antigenome represents an intermediate in genome replication, since it complexes with the N protein and acts as a template for the further synthesis of progeny genomes. Efficient replication of SeV RNA depends on the “rule of six”, which specifies that the viral genome must be of polyhexameric length in order to replicate efficiently (Calain and Roux 1993). In contrast, replication of the RSV genome does not obey the rule of six (Samal and Collins 1996). In common with other enveloped viruses, paramyxoviruses bud from the host cell plasma membrane at sites where viral components have assembled. Emerging buds then pinch off, resulting in the release of progeny virus particles.

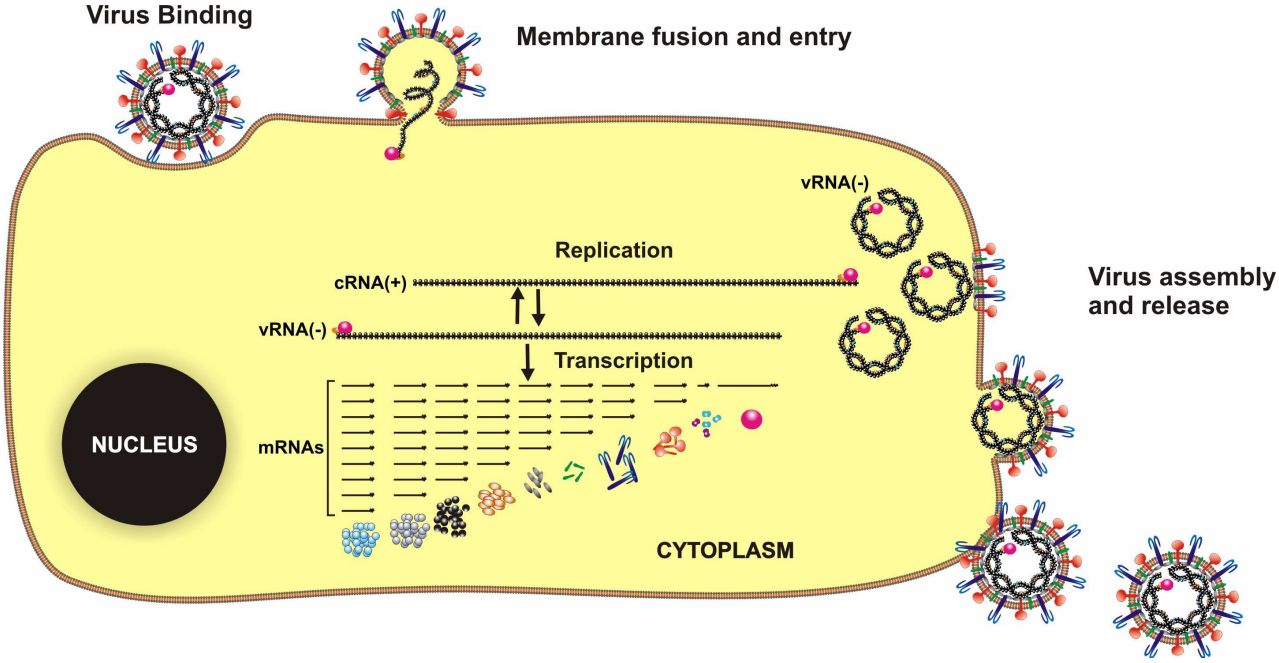


Figure 1.3. The Infectious cycle of paramyxoviruses. Schematic representation of the paramyxovirus replication cycle (see text for details). The upper part of the figure illustrates attachment, fusion and entry of the virus, resulting in release of the (-) sense nucleocapsid into the cytoplasm, where transcription and replication take place. mRNA transcripts are indicated by horizontal lines, with the gradient of decreasing molecular abundance of the mRNAs also illustrated. The right of the figure depicts assembly of viral proteins at the plasma membrane, where buds emerge and pinch off, resulting in the release of virions. Figure provided by Alfonsina Trento (ISCIII, Madrid, Spain).

1.3 Paramyxovirus envelope glycoproteins

1.3.1 The paramyxovirus fusion protein

Paramyxovirus fusion proteins mediate virus entry by fusion between the viral envelope and cellular plasma membrane at neutral pH. In addition, F proteins expressed at the plasma membrane of infected cells can fuse with neighbouring cells to form multinucleated syncytia. Despite low sequence identity, paramyxovirus F proteins share structural elements, including the location of cysteine, glycine and proline residues, suggesting a similar structure for all paramyxovirus F proteins. As shown in Fig. 1.4A, the F protein contains three principal hydrophobic regions: an N-terminal signal peptide (SP) that directs translocation to the ER during biosynthesis, a C-terminal transmembrane anchor (TM), and a fusion peptide (FP), located at the N terminus of F1. The fusion peptide inserts into the target cell membrane during membrane fusion (Russell *et al*., 2001). Paramyxovirus F proteins are also characterised by two 4-3 heptad repeat sequences, HRA and HRB, located adjacent to the fusion peptide and transmembrane domain, respectively, which play crucial roles in the mechanism of membrane fusion (see section 1.3.3).

The paramyxovirus F protein is a homotrimer (Russell *et al*., 1994) and belongs to the class I family of viral fusion proteins, which includes influenza virus haemagglutinin (HA) protein, the HIV-1 gp160 envelope protein and the Ebola virus G protein. Membrane fusion occurs via refolding of a prefusion, metastable form of the F protein (Fig. 1.4B) to a more stable, postfusion state (Fig. 1.4C) via large conformational changes (for a review see Lamb and Jardetzky 2007). Such conformational changes result in the assembly of F protein heptad repeat regions (HRA and HRB) into a 6-helix bundle (6HB), which is characteristic of the postfusion structure (Fig. 1.4C). The 6HB is a trimeric, coiled-coil structure, consisting of an internal core of three HRA helices surrounded by three anti-parallel HRB helices, (Baker *et al*., 1999; Zhao *et al*., 2000). This arrangement positions the fusion peptide and transmembrane domain at the same end of the F protein, and is thought to be directly linked to

merging of viral and cell membranes, with the free energy released on 6HB formation driving membrane fusion (Melikyan *et al*., 2000; Russell *et al*., 2001).

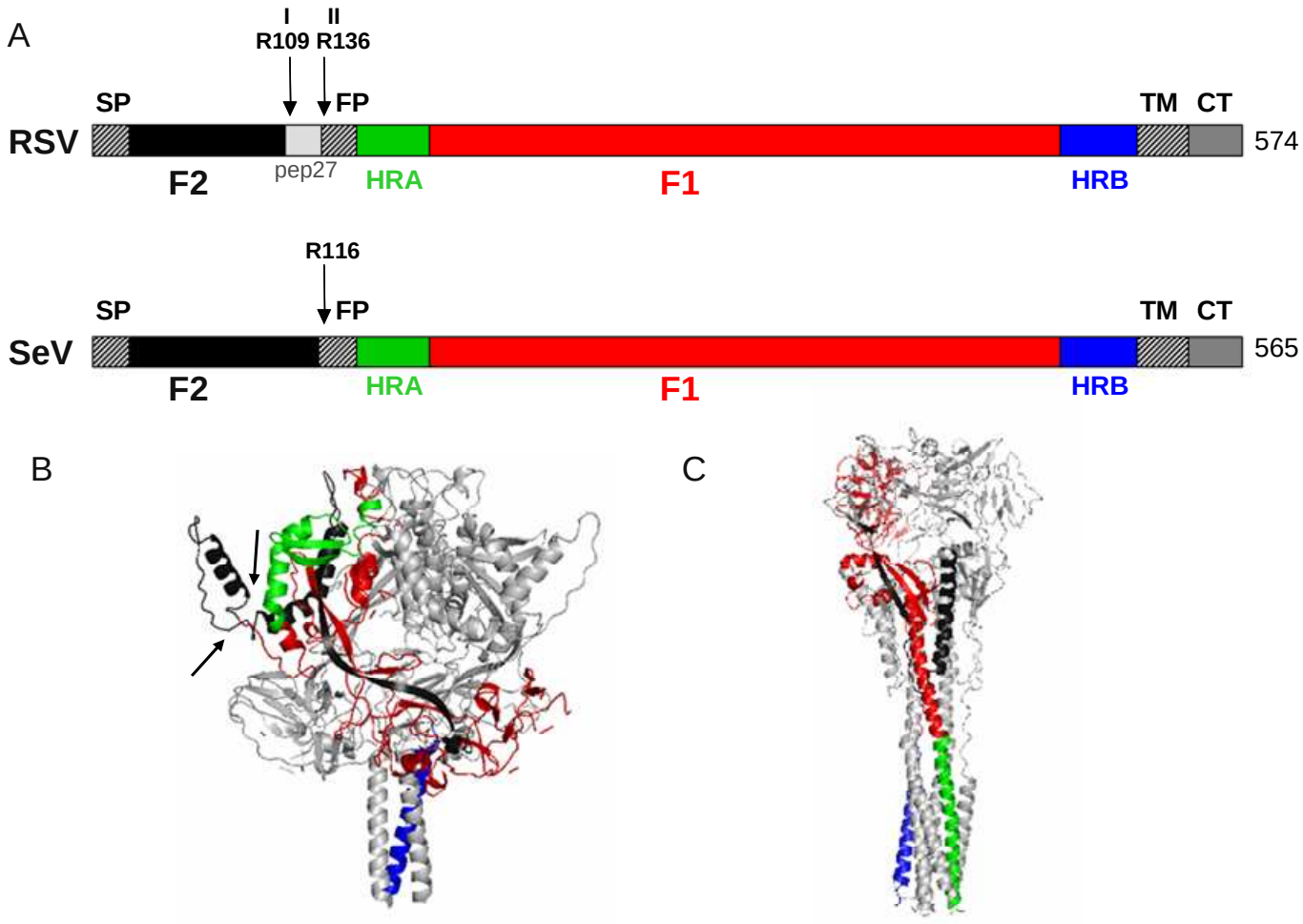


Figure 1.4. Schematic diagram of the respiratory syncytial virus (Long strain) and Sendai virus (Harris strain) fusion proteins. (A) The fusion (F) protein is synthesised as a precursor (F0), which requires proteolytic cleavage at single (F_SeV) or double (F_RSV) cleavage site(s) to generate disulphide-linked F2-F1 polypeptides. Paramyxovirus fusion proteins contain three main hydrophobic regions: the signal peptide (SP), the fusion peptide (FP) at the N terminus of F1, and the transmembrane region TM. Also shown are the heptad repeat regions A and B (green and blue, respectively). Three-dimensional models of the trimeric prefusion (B) and postfusion (C) structures of RSV F protein are shown, with cleavage sites in the prefusion form indicated by arrows. The backbone is shown in grey and a single monomer is coloured according to the same scheme as in part (A). Models were built using the SWISS-MODEL server facilities (http://swissmodel.expasy.org/) and the atomic coordinates of the prefusion structure of the parainfluenza virus 5 F protein (Protein Data Bank code 2B9B) or the postfusion structure of the parainfluenza virus 3 F protein (Protein Data Bank code 1ZTM) as templates (Yin *et al*., 2005; 2006). Figures 1.4B and 1.4C were provided by Margarita Magro (ISCIII, Madrid, Spain).

The F polypeptide is synthesised as an inactive precursor (F0), which requires proteolytic cleavage to yield fusion-competent, disulphide-linked F2-F1 polypeptides (reviewed in Lamb *et al*., 2006; Lamb and Jardetzky 2007). Cleavage takes place at either a mono- or multi-basic cleavage site, recognised by trypsin- or furin-like proteases, respectively, immediately upstream of the hydrophobic fusion peptide. Cleavage is an absolute requirement in order to prime the F protein for membrane fusion. Furthermore, the nature of the cleavage motif has been shown to act as a key determinant of viral pathogenicity, since the acquisition of a multibasic cleavage motif is linked to increased pathogenicity and systemic infection (Klenk and Garten 1994; Nagai and Klenk 1977).

A comparison of the sequences of representative paramyxovirus F protein cleavage sites is given in Table 1.1. The intracellular subtilisin-like protease furin is thought to cleave paramyxovirus proteins with multibasic cleavage sites, including the RSV F protein (F_RSV). In contrast, Sendai virus F protein (F_SeV) possess a single basic amino acid at the cleavage site (R116), and requires the addition of trypsin to cell cultures in order to undergo multiple rounds of replication (Scheid and Choppin 1974; Kido *et al*., 1999). It is thought that the trypsin-like serine protease tryptase Clara, which is secreted from Clara cells of the bronchial epithelium, is responsible for activating paramyxoviruses with monobasic cleavage sites in the murine host respiratory tract (Kido *et al*., 1997; 1999).

F_RSV is unique among the paramyxoviruses, since F_RSV possesses two (rather than one) furin cleavage sites (site I, RARR109 and site II, KKRKRR136), which are separated by a region of 27 amino acids (pep27). The two cleavage sites and the length, but not the sequence, of pep27 are highly conserved in all bovine and human RSV strains. Proteolytic processing at both cleavage sites is required by F_RSV to fuse transfected cells to form syncytia (González-Reyes *et al*., 2001; Zimmer *et al*., 2001a). Interestingly, while membrane fusion by F_RSV does not depend on co-expression of an attachment protein (see section 1.3.4), there is a requirement for double

cleavage of the F protein and removal of the intervening pep27, which is secreted into the cell culture medium (Ruiz-Argüello *et al*., 2002). While the presence of two cleavage sites in F_RSV is unique among paramyxoviruses, two cleavage sites have also been observed in the severe acute respiratory syndrome (SARS) coronavirus spike (S) protein (Belouzard *et al*., 2009).

Table 1.1. Paramyxovirus fusion protein cleavage site sequences.

Virus[1]	Cleavage Site Sequence[2]
SeV	GVPQS**R**$_{116}$ ↓
HPIV1	DNPQS**R**
HPIV3	DP**R**T**KR**
PIV5	T**RRRRR**
Mumps	S**RR**H**KR**
NDV (avirulent)	GG**R**QG**R**
NDV (virulent)	G**RR**Q**KR**
Measles	S**RR**H**KR**
RSV	**R**A**RR**$_{109}$ **KKRKRR**$_{136}$

[1]HPIV, human parainfluenza virus; PIV5, parainfluenza virus 5; NDV, Newcastle disease virus.
[2]Basic residues are indicated in boldface. Cleavage takes place at the C-terminal arginine residue (indicated by an arrow for SeV). Adapted from Harcourt *et al*., 2000.

The function of double cleavage and subsequent pep27 removal are currently unknown, although completion of cleavage is associated with a change in shape of RSV F protein (Melero 2007; Ruiz-Argüello *et al*., 2002). An immunomodulatory role has been suggested for bovine RSV (BRSV) pep27 (Zimmer *et al*., 2003), however no such role has been attributed to the human RSV intervening peptide, which does not share sequence similarity with its bovine counterpart.

The crystal structures of the pre- and post-fusion forms of three paramyxovirus F proteins have recently been solved, revealing substantial conformational differences between the two structures (Swanson *et al*., 2010; Yin *et al*., 2005;

2006). Three-dimensional models of the pre- and post-fusion forms of the RSV F protein were constructed based on the published structures for the prefusion PIV5 (Fig. 1.4B) and postfusion HPIV3 proteins (Fig. 1.4C). The overall shape of the prefusion form is that of a globular head attached to a rod-like stalk, which is formed by the triple stranded, coiled-coil adopted by HRB. The top of the globular head is formed by HRA and the fusion peptide, which is mostly buried between subunits of the globular head. The positioning of the fusion peptide resembles that of the prefusion structure of influenza virus HA (Skehel and Wiley 2000). Although its conformation is unknown, the glycosylated, intervening region between the two RSV cleavage sites (pep27) is predicted to form a solvent-accessible loop. As shown in Fig. 1.4C, the postfusion form can be related to the prefusion form by flipping the stalk domain relative to the head. During the fusion process (see section 1.3.3), the HRA region refolds to form a single, extended alpha-helical conformation and interacts with HRB to form the postfusion 6HB.

1.3.2 Paramyxovirus attachment proteins

Paramyxovirus attachment proteins (G, H or HN, depending on the virus) differ widely in their structure and receptor usage. Sendai virus, in common with other members of the *Respirovirus* and *Rubulavirus* genera, possesses an attachment protein with haemagglutinin-neuraminidase (HN) activity. Binding of HN to sialic acid-containing receptors on the cell membrane is thought to mediate attachment of the virus to the target cell, whereas HN neuraminidase activity may prevent self-aggregation of budding virus particles and facilitate virus spread (for a review, see Villar and Barroso 2006). The HN protein also promotes fusion, and co-expression of homotypic F and HN proteins is required for fusion of most paramyxoviruses (Horvath *et al*., 1992). It has been proposed that receptor binding of the HN protein induces conformational changes that activate or trigger the F protein for fusion (Russell *et al*., 2001).

The HN protein (Fig. 1.5) is a class II integral membrane protein, which is characterised by an N terminal-proximal transmembrane anchor region that acts as a signal peptide for translocation to the ER during biosynthesis, a

membrane-proximal stalk domain and a large C-terminal globular head domain that contains receptor-binding and neuraminidase activities (Thompson and Portner 1987). The structure of the HN globular head domain (Fig. 1.5B) exhibits the six-blade propeller fold typical of other neuraminidase structures (Crennell *et al*., 2000; Lawrence *et al*., 2004; Yuan *et al*., 2005). Although it was initially thought that both receptor-binding and neuraminidase activities were located at the same site, studies on both Newcastle disease virus (NDV) and SeV HN proteins suggest the presence of a second sialic acid binding site at the dimer interface (Bousse *et al*., 2004; Bousse and Takimoto 2006; Zaitsev *et al*., 2004). For NDV, it has been postulated that engagement of the first site activates the second site, thus ensuring that the virus remains in close proximity to the target cell membrane during infection (Porotto *et al*., 2006).

The HN protein forms homotetramers via the stalk region (Yuan *et al*., 2005), which may also interact with the F protein in order to promote fusion (see section 1.3.4). Indeed, studies using mutant HN proteins have demonstrated that the F protein-triggering function of HN that activates the F protein for membrane fusion resides in the HN stalk domain (Porotto *et al*., 2003). The fine balance between receptor-binding, receptor cleavage and F-triggering properties of HN is thought to regulate viral entry.

The structure of the pneumovirus attachment (G) protein (Fig. 1.5) differs widely from the paramyxovirus HN attachment protein, since G lacks either haemagglutinating or neuraminidase activitvities, and does not bind to sialic acid. The RSV G protein is a type II integral membrane protein with a single N-terminal hydrophobic signal/transmembrane anchor domain. RSV G protein is characterised by two heavily glycosylated mucin-like domains, and migrates on SDS-PAGE with a higher molecular weight than predicted due to extensive O-glycosylation. In contrast to the requirement for the paramyxovirus HN protein in fusion promotion, the pneumovirus G protein is not essential for cell-cell or virus-cell fusion (Biacchesi *et al*., 2004; Karron *et al*., 1997; Schmidt *et al*., 2002; Schowalter *et al*., 2006; Techaarpornkul *et al*., 2001; Techaarpornkul *et al*., 2002).

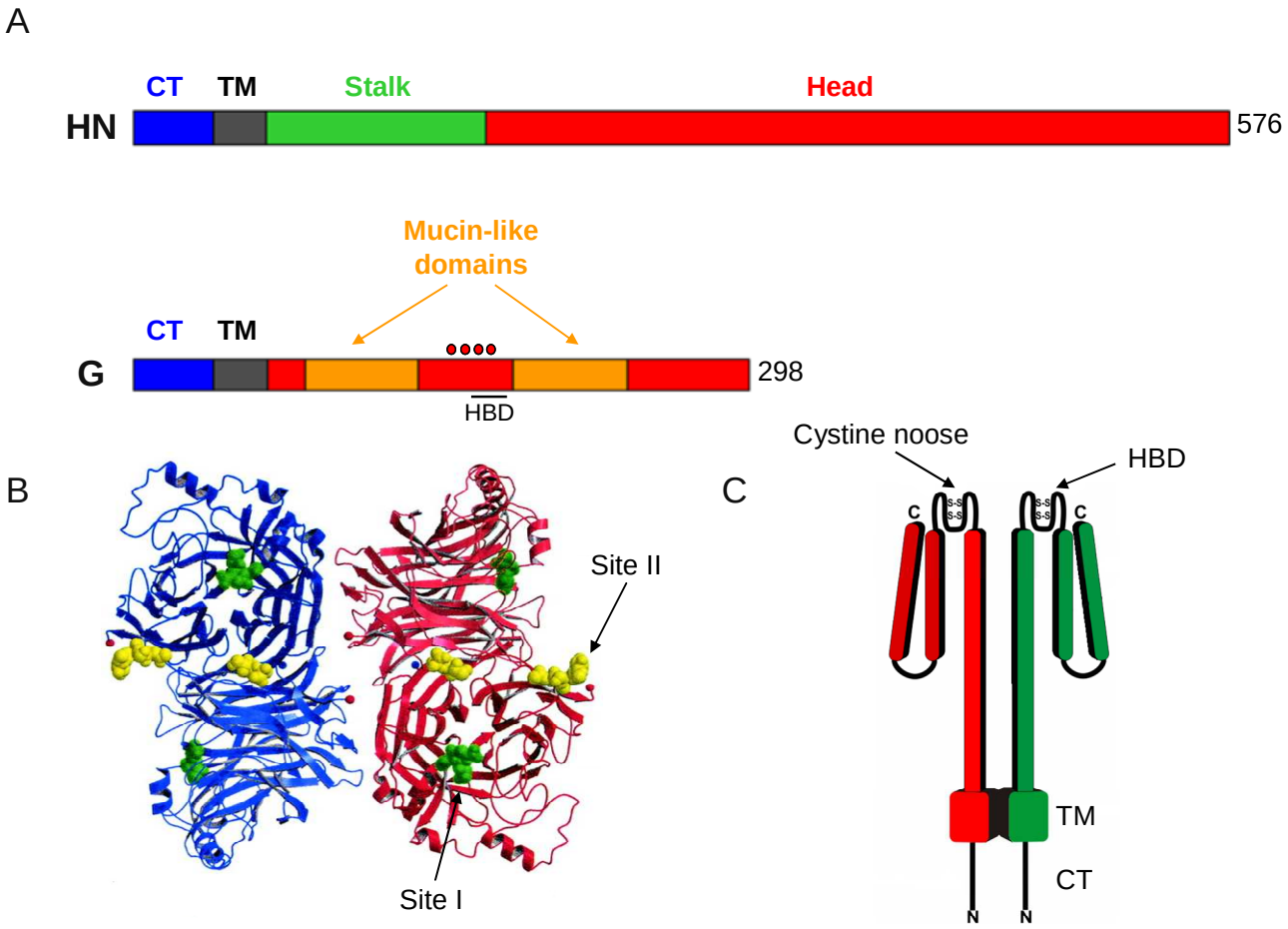


Figure 1.5. Comparison of the Sendai virus HN and respiratory syncytial virus G attachment proteins. (A) The primary structures of SeV HN (Harris strain) and RSV G (Long strain) attachment proteins are shown. Cytoplasmic tail regions (CT) and transmembrane (TM) regions are shown in blue and black, respectively. The HN stalk region, which has been proposed to interact with the prefusion F protein, is shown in green. Highly O-glycosylated, mucin-like domains of RSV G protein are indicated by arrows and cysteine residues overlapping the central conserved domain are represented by solid circles. (B) Crystal structure of the tetrameric NDV HN globular head region (Zaitsev *et al*., 2005; Protein Data Bank code 1USR). The two sialic acid binding sites, indicated by arrows, are occupied by Neu5Ac2en (2-deoxy-2,3-dehydro-N-acetylneuraminic acid), shown in green at the active site (site I), or by the thiosialoside Neu5Ac-2-S-α(2,6)Gal1OMe at site II (yellow). (C) Schematic model of the 3-dimensional structure of the transmembrane G protein (Gm). While Gm is tetrameric, a dimer is shown for simplicity. The CT and TM regions, cystine noose and the proposed heparin binding domain (HBD) are indicated. Fig. 1.5C provided by Alfonsina Trento (ISCIII, Madrid, Spain).

As shown by the model in Figure 1.5C, the RSV G mucin-like regions are predicted to form extended, unfolded domains, which are separated by a central

conserved region that is devoid of glycosylation sites. This region contains four conserved cysteine residues, which form two disulphide bridges, resulting in a cystine noose. While the central conserved region is not required for efficient replication of RSV, it may play an immunomodulatory role (Polack *et al*., 2005). A short segment adjacent to the cystine noose has been identified as a heparin binding domain (HBD), which mediates binding of the G protein to cell surface glycosaminoglycans (GAGs), (Feldman *et al*., 1999).

The G protein is produced as both (i) a type II transmembrane protein (Gm) that is incorporated into the virion envelope via a hydrophobic region near the N-terminus and (ii) by alternative translation initiation as a soluble protein (Gs), which lacks the membrane anchor region and is secreted from infected cells (Hendricks *et al*., 1987). While Gm forms homo-oligomers (probably tetramers), Gs remains monomeric, suggesting that the transmembrane region is required for oligomerisation (Escribano-Romero *et al*., 2004). While Gm is responsible for attachment to target cell GAGs during viral entry, it has been postulated that Gs may act as an antibody decoy or play an immunomodulatory role.

1.3.3 Model of paramyxovirus fusion

Earlier studies performed using peptides representing the heptad repeat regions revealed that both HRA and HRB are exposed by conformational changes of the F protein during fusion, before they refold to the final, thermostable 6 helix bundle (6HB) structure (Russell *et al*., 2001). Subsequent elucidation of crystal structures of the pre- and post fusion forms of the F protein gave rise to a hypothesised mechanism of membrane fusion, summarised in Fig. 1.6 (Yin *et al*., 2006). In the first step, the HRB helices melt and separate from the prefusion head region (Fig. 1.6B). This “open stalk” intermediate refolds to form a “prehairpin” intermediate, in which the HRA coiled-coil assembles, resulting in translocation of the fusion peptide towards the target cell membrane (Fig. 1.6C). Final refolding of the HRB region to form the 6HB results in formation of a fusion pore, which leads to membrane fusion (Fig. 1.6D). In the final, postfusion form of the F protein, both the transmembrane anchor and fusion peptide are present in the same membrane (Fig. 1.6E).

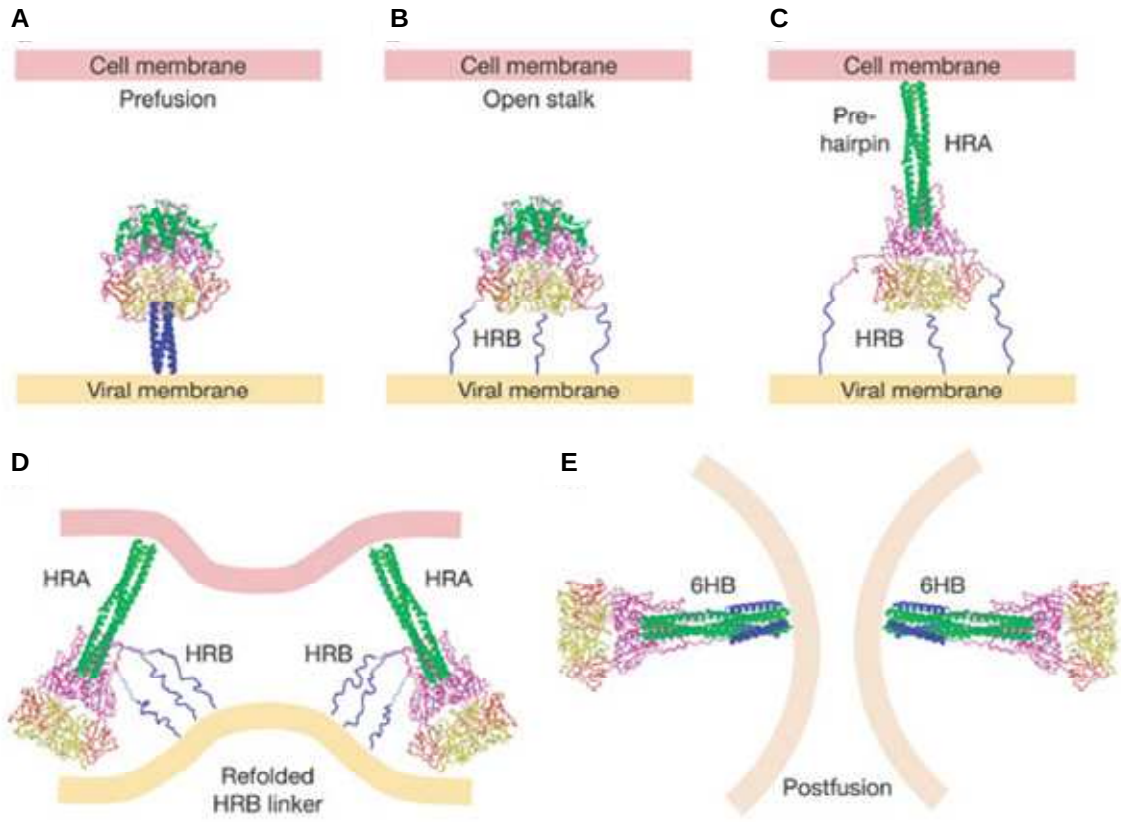


Figure 1.6. Model of paramyxovirus fusion.

(A) Structure of the prefusion conformation of the F protein. Residues corresponding to the HRA and HRB helices are coloured green and blue, respectively. (B) Open stalk conformation, in which the HRB stalk melts and separates from the prefusion head region. (C) Prehairpin intermediate, in which the HRA coiled-coil has formed, permitting insertion of the fusion peptide into the target cell membrane. (D) Close approach of the cellular and viral membranes via refolding of the HRB linker region. (E) Formation of the postfusion form of the F protein, characterised by a 6-helix bundle (6HB) and juxtaposition of the fusion peptide and transmembrane regions, which is linked to pore formation and membrane fusion. Figure reproduced from Yin *et al* 2006.

1.3.4 Role of the attachment protein in paramyxovirus fusion

It has been hypothesised that the HN protein lowers the activation energy required for F-mediated membrane fusion (Paterson *et al*., 2000) and triggers F-mediated membrane fusion “at the right time and in the right place” following binding to target cell sialic acid receptors (Lamb *et al*., 1993). An interaction between the F and HN proteins has been demonstrated on the surface of

transfected (Deng *et al*., 1999) or infected cells (Stone-Huslander and Morrison 1997). Studies conducted with chimeric HN proteins have suggested that the HN stalk and globular head regions (Deng *et al*., 1995; Tanabayashi and Compans 1996; Tsurudome *et al*., 1995) are involved in interactions with the F protein. Two models have been proposed to account for how HN may trigger F protein activation (Connolly *et al*., 2009). In the first model (termed the clamp model), HN interacts with the F protein before binding to target cells in order to stabilise the F protein in a metastable state. Conformational changes that take place in the HN protein as a result of attachment to target cell sialic acid act to release the F protein for fusion. An alternative model (the provocateur model), hypothesises that conformational changes in HN following receptor binding actively trigger fusion by destabilising the F protein. According to this second model, an interaction between HN and F could take place either before or after receptor binding. However, the nature of the conformational changes in HN and their precise effect on the F protein remain to be determined.

While Sendai virus F protein, in common with most other members of the *Paramyxoviridae* subfamily, requires co-expression of the HN attachment protein in order to fuse cells in culture, RSV F protein is able to fuse membranes in the absence of the attachment G protein. Spontaneous mutants or genetically engineered recombinant RSV expressing F as the only surface glycoprotein are able to infect cells and form syncytia (Karron *et al*., 1997; Schmidt *et al*., 2002; Techaarpornkul *et al*., 2001; Techaarpornkul *et al*., 2002). Furthermore, expression of the RSV F protein as the only viral protein in transfected cells is sufficient to induce syncytia formation (González-Reyes *et al*., 2001; Zimmer *et al*., 2001a). F_RSV binds to heparan sulphate-like glycosaminoglycans (GAGs), (Feldman *et al*., 2000; Techaarpornkul *et al*., 2002), which may mediate attachment in the absence of the G protein. The human metapneumovirus (HMPV) F protein also induces syncytia formation in transfected cells in the absence of the G protein, which is dependent on low pH for some strains (Herfst *et al*., 2009; Schowalter *et al*., 2006; 2009). Thus, the precise role in fusion of attachment proteins belonging to members of the *Pneumovirinae* subfamily is not known, and it appears that pneumovirus F proteins possess alternative mechanisms for attachment and fusion.

2. AIMS

2. AIMS

RSV is unique among the paramyxoviruses, since (a) RSV is able to fuse and infect cells in the absence of a separate attachment protein, and (b) the RSV F protein contains double furin cleavage sites, separated by a conserved intervening region (pep27). Thus, we aimed to investigate the relevance of the F_RSV double cleavage sites for fusion and infection in the context of the SeV F protein, which (a) requires co-expression of the HN attachment protein for fusion and infection, and (b) possesses a single trypsin-dependent cleavage site. We proposed the following aims:

1. Subclone SeV F and HN proteins in the pTM1 plasmid and confirm their expression in transfected BSR-T7/5 cells.
2. Construct a series of chimeric F_SeV mutants by insertion of one or both F_RSV cleavage sites in F_SeV, separated either by a partial or complete intervening pep27 sequence.
3. Compare the expression and proteolytic processing of cleavage site mutant F_SeV proteins in transfected cells.
4. Evaluate the ability of cleavage site F_SeV mutants to fuse transfected cells in the absence or presence of (a) trypsin and/or (b) the HN protein.
5. Subclone F_SeV cleavage mutants in the FL5 plasmid (SeV cDNA), and rescue recombinant SeV (rSeV) by co-transfection of BSR-T7/5 cells with FL5 and support plasmids.
6. Compare the replication rate of the distinct rSeV over multiple cycles of infection.
7. Assess the thermostability of rSeV by haemolysis and titration assays.
8. Evaluate the relative abilities of rSeV to fuse infected cells (a) in the absence or presence of trypsin and (b) at distinct temperatures.
9. Analyse the dependency of rSeV on the interaction with sialic acid receptors for cell-cell fusion and infection.

3. MATERIALS & METHODS

3. MATERIALS AND METHODS

3.1. BIOLOGICAL MATERIALS

3.1.1 Cell lines

BHK-21

Syrian golden hamster (*Mesocricetus auratus*) kidney cells. American type culture collection (ATCC): CCL-10.

BSR-T7/5

BHK-21-derived cell line that constitutively expresses the T7 bacteriophage RNA polymerase (Buchholz *et al*., 1999).

LLC-MK2

Rhesus monkey (*Macaca mulatta*) kidney cells. ATCC: CCL-7.

Pro-5

Chinese hamster ovary (*Cricetulus griseus*; CHO) cells that represent the parental line for the Lec2 glycosylation mutants. Pro-5 cells are proline auxotrophs and are thus grown in Alpha Minimum Essential Medium (AMEM), which contains 40mg/L proline. ATCC: CRL-1781.

Lec2

Sialic acid-deficient cells derived from the Pro-5 cell line by selection for resistance to wheat germ agglutinin. ATCC: CRL-1736, (Stanley *et al*., 1975).

3.1.2 Virus

Recombinant Sendai Virus (rSeV) from the Z strain was rescued from full length cDNA (FL5 plasmid), which was kindly provided by D. Kolakofsky, Geneva, Switzerland (see sections 3.1.4 and 3.3.2.4).

3.1.3 Animals

Eight-month-old BALB/c mice were used for the production of ascitic fluid expressing the GB5 monoclonal antibody directed against the SeV F protein (see sections 3.1.5 and 3.3.1).

New Zealand rabbits were employed in the production of a polyclonal serum recognising the N-terminal 25 amino acids of the SeV HN protein cytoplasmic tail (CT) region (see sections 3.1.5 and 3.3.1).

Eight-to-ten-day-old embryonated chicken eggs (La Granja, Madrid) were employed in the rescue and amplification of recombinant SeV (see section 3.3.2.4).

Dunkin-Hartley guinea pigs were bled at two-week intervals for haemolysis experiments (see section 3.3.5.3) and stored at 4ºC in Alsever's solution (one part blood to one part Alsever's).

3.1.4 Bacteria and Plasmids

Escherichia coli DH5α strain (Hanahan *et al*., 1983) or XL1-Blue competent bacteria (Stratagene) were cultivated in the following media supplemented with 100 µg/ml ampicillin:

LB medium (MP Biomedicals: 1% tryptone, 1% NaCl and 0.5% yeast extract).

NZY+ (1% casein hydrolysate, 0.5% yeast extract [Becton Dickinson], 0.5% NaCl, 12.5 mM $MgCl_2$, 12.5 mM $MgSO_4$, 20 mM glucose).

Circle-Grow® solid media (Qbiogene).

The following plasmids were employed in cloning and mutagenesis (see section 3.3.3):

pGEM4

2871 bp plasmid carrying T7 and SP6 promoters. The genes encoding Sendai virus F and HN proteins were originally provided in the pGEM4 plasmid by L. Roux, Geneva, Switzerland.

pTM1

5357 bp plasmid containing the T7 promoter and the encephalomyocarditis virus (ECMV) UTR, which increases the efficiency of translation (Moss *et al*., 1990). SeV F and HN genes were subcloned from the pGEM4 plasmid into pTM1, and subsequently subjected to mutagenesis (section 3.3.3).

FL5

18294 bp plasmid (a gift from D. Kolakofsky, Geneva, Switzerland), which contains the complete SeV cDNA (Z strain) and a number of unique restriction sites. Mutant SeV F genes (Harris strain) were cloned into the FL5 plasmid, and recombinant SeV was rescued as detailed in section 3.3.2.4.

3.1.5 Antibodies

GB5

Monoclonal antibody (MAb) that recognises the SeV F protein F1 polypeptide. GB5 was obtained from the ascitic fluid of BALB/c mice injected with GB5 hybridoma cells (a gift from S. Wharton and J.J Skehel, London, UK). See section 3.3.1.

2F

MAb that recognises the RSV F protein (García-Barreno *et al*., 1989).

F_SeV_CT

Polyclonal serum recognising the cytoplasmic tail (CT) of F_SeV (a gift from L. Roux, Geneva, Switzerland).

HN_SeV_CT

Polyclonal serum that recognises the CT of HN_SeV was prepared by inoculation of New Zealand rabbits with a peptide corresponding to the N-terminal 25 amino acids of the HN CT (see section 3.3.1).

F_RSV_104-117

Polyclonal serum that recognises a peptide corresponding to amino acids 104-117 of the RSV F protein (González-Reyes *et al*., 2001).

3.1.6 Oligonucleotides

Table 3.1 Oligonucleotides employed in this study

Oligonucleotide[a]	Use	Sequence
F_NcoI+	Cloning of SeV F in pTM1	GCACATGCCATGGACAGCATATATCCAGAGGTCAC
F_SpeI-	Cloning of SeV F in pTM1	GCTCAGGACTAGTTCATCGTTTCTCAGCCATCGCA
HN_NcoI+	Cloning of SeV HN in pTM1	GCACATGCCATGGATGGTGATAGGGGCAAACG
HN_SpeI-	Cloning of SeV HN in pTM1	GCTCAGGACTAGTTTAAGACTCGGCCTTGCATA
G_NcoI+	Cloning of RSV G in pTM1	GCACATGCCATGGTCCAAAAACAAGGACCAACGCACCGC
G_SpeI-	Cloning of RSV G in pTM1	GCTCAGGACTAGTCTACTGGCGTGTTGTGTTGGGTGG
F_MluI+	Cloning of SeV F in FL5	AACTTAGGGATAAAGTCCCTTGTGA*AC*GCGTGGTTGCAAAACTCTCCCCTTGGGAAACATGACAGCATATATCCAGAGGTCACAGTGC
F_RsrII-	Cloning of SeV F in FL5	TAAGTTTTTCTTATTATATACAGATCGGACCGAATACCATGCCTGCTTTACAAGACATCTGATAATGGTCGTGA**AGT**TCATCGTTTCTCAGCCATCGCATCAAACC
F_RKR+	Cleavage site[b] mutation of SeV F	GCCGGTGTTCCACAGTCGAAGAAAAGGAAAAGAAGATTCTTCGGTGCTG
F_KKRKR+	Cleavage site mutation of SeV F	GCCGGTGTTCCACAGTCGAAGAAAAGGAAAAGAAGATTCTTCGGTGCTG
F_110+	Cleavage site mutation of SeV F	GATCTTCAGGAGGCTCTGATAACTGCAAACAATCGAGCCAGAAGAGAAGTCACCAATGATACGACACAAAATGCC
F_117+	Cleavage site mutation of SeV F	CTGCAAACAATCGAGCCAGAAGAGAACTACCAAGGTTTATGAATTACACCAATGATACGACACAAAATGCCGGTG

F_Δ+	Cleavage site mutation of SeV F	GCCAGAAGAGAACTACCAAGGTTTATGAATTACACCTCGAAGAAAAGGAAAAGAAGATTCTTCGGTGC
F_130+	Cleavage site mutation of SeV F	GCCAGAAGAGAACTACCAAGGTTTATGAATTACACCCTCAACAATACCAAAAAAACCAATGTAACATTATCGAAGAAAAGGAAAAGAAGATTCTTCGGTGC
F_231-	Sequencing of SeV F	CAGCCTGTTCAGTAGGCTCTTGTAC
F_590-	Sequencing of SeV F	CAGTCTTAAGGCAGCAGTCTCACAGCCT
F_663+	Sequencing of SeV F	CGGCTCGAATTTCGGAACCATCGGA
F_1009+	Sequencing of SeV F	GATTGTGTTGAGTCCAGATTGACCT
F_1460+	Sequencing of SeV F	CCTCTCTGAGGTAGGTAGATGGT
FL5+	Sequencing of Sev F (in FL5)	GTGCACCCATCAGAGACCTGCGACAATGC
HN_274-	Sequencing of SeV HN	CTTGCGATAACCTCTTGCCT
HN_642+	Sequencing of SeV HN	AGGTTGTGCTGACATAGGGA
HN_721-	Sequencing of SeV HN	TGGGACACTACGGGGTTAAG
HN_1089+	Sequencing of SeV HN	CACATGCAATGAGGCTCTG
HN_1509+	Sequencing of SeV HN	CAACCCAACAATCATGTATT
OG_551+	Sequencing of RSV G	ATCCAACCTGCTGGGCTATCT
OG_788+	Sequencing of RSV G	TCACAAGTCAAATGGAAA

[a]The positive (+) sign indicates oligonucelotides with the same polarity as the target cDNA, whereas the negative (-) sign indicates oligonucelotides of opposite polarity. [b]For oligonucleotides employed in cleavage site mutation, the complementary (-) oligonucleotide is not shown.

3.1.7 Enzymes

The QuikChange® Site Directed Mutagenesis kit (Stratagene) was used in order to generate the F_SeV mutants detailed in Fig. 4.3.

Automated DNA sequencing kit Big Dye 3.1 (Abi Prism, Biosystems) was employed in the sequencing of viral genes.

Restriction enzymes used in cloning (Nco I, Spe I, Mlu I and Rsr II) were obtained from Roche.

Other enzymes employed in the manipulation of nucleic acids are as follows: shrimp alkaline phosphatase (USB, GE Healthcare), T4 DNA ligase (Rapid Ligation Kit, Roche), TaqPlus Long^{TM} DNA polymerase (Stratagene), Phusion® High Fidelity DNA polymerase (Finnzymes) and the OneStep RT-PCR kit (Qiagen).

Trypsin and neuraminidase (from *Clostridium perfringens*) were purchased from Sigma.

3.1.8 Eukaryotic Cell Culture media

Dulbecco's Modified Eagle Medium (DMEM; Dulbecco and Freeman 1959, Gibco), used for the cultivation of BSR-T7/5 and LLC-MK2 cells, was supplemented with 4 mM glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin and enriched with 2.5% (DMEM-2.5%) or 10% (DMEM-10%) foetal heat-inactivated calf serum (FCS).

Alpha Minimum Essential Medium (AMEM, Sigma), used for the cultivation of Pro-5 and Lec2 cells, was supplemented with 4 mM glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin and enriched with 2.5% FCS (AMEM-2.5%) or 10% FCS (AMEM-10%).

OPTIMEM reduced-serum medium used in transfection experiments was obtained from Gibco, Invitrogen.

Trypsin-Versene (0.05% trypsin, 0.02% EDTA in PBS).

Alsever's solution (20.5g Dextrose, 8g Sodium citrate, 0.55g Citric acid, 4.2g NaCl, dissolved in 1 litre of water) is an isotonic, anticoagulant blood preservative, which was used for the storage of guinea pig blood.

3.2 REAGENTS

All organic reagents (methanol, ethanol, acetone etc) were obtained from VWR.

The Wizard® Plus SV Minipreps DNA Purification System (Promega) and the SNAP Midiprep kit (Invitrogen) were used to prepare plasmid DNA. RNA extraction from cell culture supernatant was carried out using the QIAmp Viral RNA Mini Kit (Qiagen).

Electrophoresis reagents: acrylamide, sodium dodecyl sulphate (SDS), ß-mercaptoethanol, N-N-N'-N'-tetramethyl ethylenediamine (TEMED), bromophenol blue, the Precision Plus Protein Standards Dual Color molecular marker, Tris/Glycine/SDS (25 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.4) and Tween-20 were obtained from Bio-Rad. Agarose (Conda laboratories, Pronadisa) and the Benchtop 1 kb DNA ladder (Promega) were employed in nucleic acid separation.

For Western blotting experiments, Immobilon-P (Millipore) and the Amersham ECL Advance Western Blotting Detection Kit (GE Healthcare) were employed in immunotransfer and detection of immobilised antigens, respectively.

Anti-mouse/rabbit antibodies conjugated to fluorescein isothiocyanate (FITC) or peroxidase, biotinylated anti-mouse Ig and horseradish peroxidase-conjugated streptavidin were all purchased from GE Healthcare. Streptavidin-R-phycoerythrin (streptavidin-RPE) was purchased from Southern Biotechnology Associates Inc. CNBr-activated Sepharose 4B and protein A-Sepharose CL4B were obtained from Pharmacia.

Dimethylsulphoxide (DMSO), ampicillin, G418 antibiotic, pristane (2,6,10,14-tetramethylpentadecane), HT (hypoxanthine-thymidine), biotin and AEC (3-amino-9-ethylcarbazole) were obtained from Sigma.

Extraction of cellular membrane proteins was carried out using the ProteoExtract® Native Membrane Protein Extraction Kit (Calbiochem, Bionova).

For inoculation of New Zealand rabbits, Freund's Complete Adjuvant and Freund's Incomplete Adjuvant were obtained from Difco.

Eukaryotic cells were transfected using the FuGENE® HD Transfection Reagent (Roche). Cellular luciferase activity was detected using the Luciferase Assay System from Promega.

Concentration of cell culture supernatant was achieved using 100 000 MW Vivaspin 2 ml spin columns (Vivascience, Sartorius).

3.3 METHODS

3.3.1 Antibody production

HN_SeV_CT

Polyclonal HN serum was obtained by intramuscular (i.m.) injection of New Zealand rabbits with 1 mg of a peptide (corresponding to the N-terminal 25 amino acids of the SeV HN cytoplasmic tail region), in Freund's complete adjuvant. Inoculation was repeated a further two times in Freund's incomplete adjuvant at one-month intervals. Following the final inoculation, rabbits were bled a total of four times at three-week intervals.

GB5

Ascitic fluid was obtained by intraperitoneal injection (i.p.) of 0.5 ml Pristane in eight-month-old BALB/c mice, followed by i.p injection of $5x10^6$ GB5 hybridoma cells in 0.2 ml PBS eight days later. When tumour growth was observed (one week following injection), ascitic fluid was drained and clarified by centrifugation.

GB5 MAb was purified from ascitic fluid by immunoaffinity chromatography on a protein A-Sepharose CL4B column (Pharmacia), and eluted in buffer containing 1.5 M glycine, 3 M NaCl pH 8.9.

Purified GB5 (1 mg) was subsequently dialised overnight at 4ºC (0.1 M Na_2CO_3, 0.15 M NaCl pH9) and biotinylated for use in Western blotting (see section 3.3.4.3). Biotin (Sigma) was dissolved in DMSO (1 mg/ml), and 100 µl were incubated with dialysed GB5 for 2 h at room temperature (RT) with shaking. Biotinylated GB5 was subsequently dialysed against PBS and optical density at 280 nm measured to determine antibody concentration.

3.3.2 Cultivation of cells and viruses

3.3.2.1 Cultivation of bacteria

XL1-Blue competent bacteria (Stratagene) and *Escherichia coli* DH5α bacteria were employed as vectors for the production of plasmid DNA. *Escherichia coli* DH5α were prepared according to the method of Hanahan *et al*., (1983), and transformed by thermal heat shock (15 min on ice, 1 min at 42ºC, 2 min on ice). Transformation of XL1-Blue competent bacteria (Stratagene) was carried out by incubation for 30 min on ice, 45 sec at 42ºC and 2 min on ice. Bacteria were subsequently incubated for 1 h at 37ºC with shaking in NZY+ medium (XL1-Blue) or LB medium (DH5α) and plated on Circle-Grow® solid medium plates, supplemented with 100 µg/ml ampicillin. Circle-Grow® plates were incubated for 16 h at 37ºC to allow the growth of bacterial colonies. Individual colonies were selected and grown in LB medium supplemented with 100 µg/ml ampicillin for 16 h at 37ºC. DNA was purified from bacterial cultivates using the Wizard® Plus SV Minipreps DNA Purification System (Promega) or the SNAP Midiprep kit (Invitrogen).

3.3.2.2 Cultivation of eukaryotic cells

Eukaryotic cells were maintained in 100 mm dishes in DMEM-10% and incubated at 37ºC in an atmosphere of 5% CO_2 and 98% humidity. In order to passage eukaryotic cells, trypsin-versane was employed to lift the cell monolayer. The antibiotic G418 was supplemented to the medium of BSR T7/5 cells on alternate passages in order to select for cells expressing T7 polymerase. Hybridoma cells were maintained in RPMI medium supplemented

with 15% FCS and hypoxanthine-thymidine (HT). Cells were stored in liquid nitrogen in FCS supplemented with 10% DMSO.

3.3.2.3 Transfection of eukaryotic cells

Cells were grown to 90-100% confluence and transfected in DMEM-2.5% using FuGENE® HD (Roche). Transfection mixtures were prepared in OPTIMEM reduced-serum medium using a ratio of 4:2 (µl FuGENE® HD:µg DNA), according to the manufacturer's instructions. The transfection mixture was incubated for 30 min at room temperature before being added dropwise to cells. Seven hours post-transfection, the transfection mixture was removed and cells were incubated in serum-free medium with or without 0.25 µg/ml trypsin (Sigma). Extracts of transfected cells were prepared as described in section 3.3.2.7.

3.3.2.4 Rescue of Sendai virus

BSR-T7/5 cells growing in 35 mm dishes were co-transfected with 3.5 µg full-length Sendai virus cDNA (FL5), 1 µg pTM1-N, 1 µg pTM1-P and 0.35 µg pTM1-L using FuGENE® HD (see section 3.3.2.3). Twenty four hours post-transfection, cells were washed with serum-free DMEM and 0.5 µg/ml trypsin was added to the culture medium. Cells were incubated for a further 24 h at 33℃ to allow virus growth, and fresh trypsin added at 48 h post-transfection. Cells were collected at 72 h post transfection in the culture medium and injected into nine-day-old embryonated chicken eggs, which permit the growth of SeV to high titres (Garcin *et al*., 1995). Following incubation for 48 h at 34ºC, allantoic fluid was collected and clarified by low speed centrifugation. In order to confirm virus rescue, allantoic fluid was pelleted through a 25% glycerol cushion for 30 min at 15,000 rpm and loaded onto a 10% SDS-PAGE gel to detect viral proteins. Alternatively, clarified allantoic fluid was purified through a 4 ml 20% sucrose cushion in Beckman SW28 tubes by centrifugation for 2 h at 26,000 rpm, as previously described (Loney *et al*., 2009).

3.3.2.5 Cultivation of Sendai virus

LLC-MK2 cells were grown to 90-100% confluence and infected with purified Sendai virus in a small volume of DMEM-2.5% medium (just enough to cover the monolayer). Cells were incubated at 33℃ or 37° C for two hours to allow virus absorption, before being washed in serum-free DMEM. Cells were incubated in serum-free medium with or without 0.3 µg/ml trypsin until cytopathic effect was observed. In order to follow the replication of SeV over multiple replication cycles (multi-step growth curve), LLC-MK2 cells were infected at an MOI of 0.05. Over a period of 96 h, 500 µl aliquots of cell culture supernatant were taken at 24 h intervals, and replaced with fresh medium with or without 0.3 µg/ml trypsin. The supernatant from infected cells was subsequently titrated as described in section 3.2.2.6.

3.3.2.6 Titration of Sendai virus

SeV was titrated by immunohistochemical staining, according to the method of Borisevich *et al*., (2008). LLC-MK2 cells in 96-well plates ($5x10^4$ per well) were infected with 50 µl of serial dilutions of purified SeV (or supernatant from SeV-infected cells) in DMEM-2.5%. Cells were incubated at 37℃ for two hours to allow virus absorption, before being washed in serum-free DMEM. Cells were subsequently incubated overnight in serum-free medium supplemented with 0.3 µg/ml trypsin. Twenty hours post-infection, cells were washed with PBS containing 1% bovine serum albumin (PBS-1% BSA), and fixed for 30 min at 4℃ with methanol containing 2% H_2O_2. Fixed cells were blocked with PBS-1% BSA and stained for expression of SeV F protein with MAb GB5 for 1 h at room temperature, followed by incubation with anti-mouse immunoglobin conjugated to peroxidase for 30 min at room temperature. The resulting immune complexes were developed using AEC substrate in DMSO (3.3 mg/ml), dissolved in a buffer containing 0.1 M citrate, 0.2 M phosphate pH 5.5 and 0.06% H_2O_2. The AEC substrate generates an insoluble precipitate on reaction with peroxidase (Graham *et al*., 1965), resulting in red colouration of infected cells that permits their quantification by light microscopy.

3.3.2.7 Preparation of cell extracts

Extracts of membrane proteins from transfected cells were prepared using the ProteoExtract® Native Membrane Protein Extraction Kit (Bionova, Calbiochem), according to the manufacturer's instructions. Briefly, $2x10^6$ cells were resuspended in 1 ml extraction buffer and incubated for 15 minutes at 4℃ in the presence of protease inhibitors. Following centrifugation, the insoluble pellet (enriched in membrane proteins), was resuspended in 0.5 ml of an extraction buffer containing detergent for 30 minutes at 4℃, an d clarified by centrifugation.

3.3.3 Nucleic acid techniques

3.3.3.1 Cloning

The pTM1 plasmid (Elroy-Stein *et al*., 1989), carrying the full-length cDNA insert of the RSV F gene under transcriptional control of the T7 promoter (pTM1-F_RSV), has been described previously (González-Reyes *et al*., 2001).

The SeV F and HN genes were amplified from pGEM-F and pGEM-HN plasmids with TaqPlus Long™ DNA polymerase (Stratagene), according to the manufacturer's instructions, using G_NcoI+ and G_SpeI- oligonucleotides (Table 3.1), and the following PCR program (94ºC, 2 min [94 ºC 30 sec, 45 ºC 1 min, 72 ºC 5 min] x 25 cycles, 72 ºC 5 min). Amplified PCR product (50 µl) was precipitated by incubation for 1 h at -80ºC with 10 µl sodium acetate and 150 µl ethanol. Precipitated DNA was pelleted by centrifugation (13,000 rpm, 15 min at 4ºC) and the pellet was washed with ethanol (75%). Pellets were dried for 15 min at RT and resuspended in 50 µl water. Five micrograms of pTM1, F_SeV or HN PCR products were digested with 90 U Nco I (Roche) for 90 min at 37ºC and subsequently precipitated, as described above. A second digestion with 90 U Spe I (Roche) was carried out by incubation at 37ºC for 90 min, followed by a 20 min incubation at 65ºC to inactivate Spe I. Digested pTMI plasmid was incubated for 1 h at 37ºC with 2 U shrimp alkaline phosphatase (USB, GE Healthcare) to prevent re-circularisation, followed by a 20 min incubation at 65ºC to inactivate the phosphatase. Ligation of digested pTM1 plasmid and PCR products was carried out for 5 min at a 1:10 ratio using the Roche Rapid

Ligation Kit. *E.coli* DH5α competent bacteria were transformed with 2 µl ligation mix, according to the method described in section 3.3.2.1 and plated onto CircleGrow® plates. Following incubation of plates for 16 h at 37ºC, selected colonies were grown in LB medium at 37ºC for 16 h and DNA extracted using the Wizard® Plus SV Minipreps DNA Purification System (Promega). Subcloning of SeV F and HN genes in pTM1 was confirmed by DNA sequencing. Sequencing reactions were carried out using the Big Dye 3.1 Kit (Applied Biosystems), 500 ng of DNA, 30 ng of the sequencing oligonucleotides listed in Table 3.1 and the following PCR conditions (94ºC 3 min, [96ºC 30 sec, 55ºC 4 min] x 25 cycles).

The RSV G gene was subcloned into pTM1 by the same method, with the exception that reduced temperatures were employed. Incubation of transformed DH5α bacteria was carried out in LB for 5 h at 25ºC, CircleGrow® plates were grown at RT for 4 days, and selected colonies were subsequently grown in LB medium at 25ºC for 48 h.

SeV wild-type F (Harris strain) or F_SeV cleavage mutants were cloned into the FL5 plasmid by the method above, using F_MluI+ and F_RsrII- oligonucleotides (Table 3.1). According to the “rule of six”, replication of the SeV genome occurs efficiently only when the genome is an exact multiple of six nucleotides (Calain and Roux 1993). Thus, on replacement of the wild-type F_SeV gene present in FL5 with the cleavage site mutants, insertion of 3 nucleotides in the non-coding region of SeV (shown in bold in Table 3.1), was required to ensure that the length of SeV remained a multiple of six. F_SeV genes were amplified by PCR using the Phusion® High Fidelity DNA polymerase (Finnzymes), according to the manufacturer’s instructions, and the following PCR program (98ºC 30 sec [98 ºC 15 sec, 65 ºC 30 sec, 72 ºC 4 min] x 30 cycles, 72 ºC 7 min). Amplified PCR products were subsequently digested with Mlu I and Rsr II restriction enzymes (Roche) as described above, and ligated with digested FL5 in order to replace the wild-type SeV F gene (Z strain) present in FL5. XL1-Blue competent bacteria (Stratagene) were transformed with 5 µl ligation mix, according to the method described in section 3.3.2.1, with the exception that selected colonies were grown at reduced temperatures (33ºC).

3.3.3.2 Mutagenesis

The pTM1-F_SeV plasmid was subjected to mutagenesis using the QuikChange® site directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. The mutants shown in Figure 4.3 were sequentially generated using the corresponding oligonucleotides detailed in Table 3.1 (section 3.1.6), and the following PCR program (95ºC 30 sec [95 ºC 30 sec, 55ºC 1 min, 68 ºC 14 min] x 18 cycles). All mutations were confirmed by automated sequencing of the complete F gene, as described in section 3.3.3.1.

3.3.3.3 RNA extraction and RT-PCR

LLC-MK2 cells were infected in 35 mm dishes with rSeV at an MOI of 0.05 and incubated in the presence of 0.3 µg/ml trypsin trypsin for 72 h at 37ºC. The clarified supernatant of infected cells was concentrated 15-fold by centrifugation through Vivaspin 2 ml spin columns (Sartorius). RNA was subsequently extracted from concentrated supernatants using the QIAmp Viral RNA Mini Kit (Qiagen) according to the manufacturer's instructions. In order to amplify the complete F_SeV gene, RT-PCR was performed on extracted RNA using the FL5+ and HN_274- oligonucleotides (Table 3.1), and the Qiagen OneStep RT-PCR Kit (50ºC 30 min, 95ºC 15 min [94 ºC 1 min, 55 ºC 1 min, 72 ºC 2 min] x 30 cycles, 72 ºC 1 min).

3.3.4 Immunochemical techniques

3.3.4.1 Indirect immunofluorescence

Cells were grown in microchamber culture slides to 90-100% confluence and transfected with 0.5 µg total DNA, or infected with MOI 1-5. Cells were fixed 20-48 h post-infection with cold (-20ºC) methanol for 5 min, followed by cold (-20ºC) acetone for 30 seconds. Fixed cells were immunostained using MAb GB5 directed against F_SeV, followed by incubation with anti-mouse FITC-linked antibody (GE Healthcare UK Limited). Cells were examined using a Zeiss microscope and photographed using an AxioCam HRC digital camera and Axiovision 3.1 software.

3.3.4.2 Flow cytometry

Transfected BSR-T7/5 cells were detached 24 h post-transfection using 1 mM EDTA in Ca^{2+}- and Mg^{2+}-free PBS, and resuspended in DMEM (2% FCS). $3x10^5$ cells were incubated for 30 min at 4^oC with monoclonal antibody GB5 (F_SeV) or 2F (F_RSV). After pelleting and washing the cells two times with PBS, cells were stained by subsequent 30 min incubations with biotinylated anti-mouse Ig (GE Healthcare UK Limited) and streptavidin-RPE (Southern Biotechnology Associates Inc). Finally, cells were fixed in 1% paraformaldehyde and the fluorescence of $2x10^4$ cells determined using a Becton Dickinson FACSCalibur instrument with CellQuest software. Data was analysed using Flow Jo software (Tree Star, Inc).

3.3.4.3 Immunoprecipitation and Western blotting

BSR-T7/5 cells were transfected as previously described in section 3.3.2.3. Twenty four hours post-transfection, cells were washed with PBS and extracts of membrane proteins prepared using the ProteoExtract® Native Membrane Protein Extraction Kit (Calbiochem), as described in section 3.3.2.7. For the immunoprecipitation experiments, purified GB5 antibody was bound to CNBr-activated Sepharose 4B (Pharmacia), according to the manufacturer's instructions. 1 ml of membrane protein extract was incubated at 4°C with 50 µl of GB5-conjugated Sepharose. After an overnight incubation, Sepharose beads were washed four times with PBS, resuspended in 50 µl sample buffer, (80 mM Tris, 2% SDS, 10% glycerol and 0.01% bromophenol blue), and boiled for 6 minutes. Following centrifugation to remove beads from the eluted proteins, 5% β-mercaptoethanol was added and the immunoprecipitated proteins were separated on a 10% acrylamide SDS-PAGE gel. Proteins were transferred to an Immobilon membrane (Millipore), and subjected to Western blotting in order to detect F_SeV proteins using biotinylated GB5 monoclonal antibody and streptavidin-horseradish peroxidase conjugate (GE Healthcare UK Limited). Bands were visualised using the Amersham ECL Advance Western Blotting Detection Kit (GE Healthcare UK Limited), and imaged using a VersaDoc camera and Quantity One 1D analysis software (Bio-Rad Laboratories).

Supernatants from infected LLC-MK2 cells were separated on a 10% SDS-PAGE gel, transferred to an Immobilon membrane (Millipore), and subjected to Western blotting using the polyclonal rabbit sera F_RSV_104-117 or F_SeV_CT (see section 3.1.5). Membranes were subsequently incubated with anti-mouse immunoglobin conjugated to peroxidase. Bands were developed using the Amersham ECL Advance Western Blotting Detection Kit and imaged using a Kodak Gel Logic 1500 Imaging System camera and Kodak Molecular Imaging software. The net intensity of protein bands (indicated in the legend to Fig. 4.11) was measured using Kodak Molecular Imaging software, and the percentage of F protein cleavage to F1 was calculated by dividing the intensity of F1 bands by the total net intensity.

3.3.4.4 Enzyme Linked Immunosorbant Assay (ELISA).

Infected Pro-5 or Lec-2 cells (MOI 1) growing in 96-well microtitre plates were washed 20 h post infection with PBS containing 0.05% Tween-20 (PBS-Tween, Bio-Rad Laboratories) and fixed in 80% acetone. The wells were blocked for 30 min at room temperature with 5% pig serum in 0.05% PBS-Tween. Cells were subsequently stained for expression of SeV F protein with MAb GB5 for 1h at room temperature, followed by incubation with anti-mouse Ig conjugated to horseradish peroxidase (GE Healthcare UK Limited) for 45 min at room temperature. Optical density at 490 nm was measured after the addition of the substrate o-phenylene diamine (OPD), dissolved in a buffer containing 0.1 M citrate, 0.2 M phosphate pH 5.5 and 0.06% H_2O_2.

3.3.5 Fusion assays

3.3.5.1 Syncytium formation assay

The size of syncytia in infected LLC-MK2 cells growing in microchamber slides (see section 3.3.4.1) was measured by counting the number of nuclei in 10 syncytia selected at random from distinct 20x magnification fields. For the experiment shown in Fig. 4.15, LLC-MK2 cells were pre-treated prior to infection with 120 mU neuraminidase from *Clostridium perfringens* (Sigma) in OptiMEM medium (Gibco) for 90 min at 37ºC. The level of infection in the absence or presence of neuraminidase was determined by counting the number of infected cells in five 20x magnification fields selected at random.

3.3.5.2 Luciferase reporter gene assay

BSR-T7/5 cells were transfected in 48-well plates with 0.5 μg total DNA. BHK cells in 100 cm dishes were simultaneously transfected with 30 μg of a plasmid encoding the luciferase gene (pTM1-Luc). For the cell-cell fusion assay, BHK cells were detached 7 h post-transfection using 1 mM EDTA in Ca^{2+}- and Mg^{2+}-free PBS, resuspended at a density of $1x10^6$ cells/ml in serum-free medium, and overlaid at a 1:1 ratio onto BSR cells, either in the presence or absence of 0.25 μg/ml trypsin (Sigma). The mixed donor and target cells were subsequently incubated at 37ºC to allow fusion. At 30 h post-transfection, (or 48 h post transfection for experiments involving F_RSV), cells were washed with PBS and lysed in 1x Passive Lysis Buffer (Promega Inc), according to the manufacturer's instructions. 10-20 μl of clarified cell extract were mixed with 100 μl luciferase assay substrate (Promega Inc), and immediately assayed for luciferase activity over a 10 second measurement read using a Turner Biosystems 20/20n Luminometer instrument. Background activity (subtracted from all results shown), was determined by overlaying BHK cells transfected with pTM1-Luc onto BSR cells, which had previously been transfected with the pTM1 plasmid.

Alternatively, BSR-T7/5 cells were infected in DMEM-2.5% at an MOI of 5 and incubated at 37℃ for two hours. BSR-T7/5 cells were subsequently washed in serum-free DMEM and incubated for 14 h in serum-free medium supplemented

with or without 0.25 µg/ml trypsin. LLC-MK2 cells in 100 cm dishes were transfected (FuGENE® HD, Roche) with 30 µg of a plasmid encoding the luciferase gene (pTM1-Luc), and incubated at 37°C for 14 h in DMEM-2.5%. For the cell-cell fusion assay, LLC-MK2 cells were detached 14 h post-transfection using 1 mM EDTA in Ca^{2+}- and Mg^{2+}-free PBS, resuspended at a density of $1x10^6$ cells/ml in serum-free medium, and overlaid at a 1:1 ratio onto BSR-T7/5 cells, either in the presence or absence of 0.25 µg/ml trypsin (Sigma). Mixed donor and target cells were incubated for 3 h at 33°C or 37°C to allow fusion. Cells were subsequently washed with PBS, lysed in 1x Passive Lysis Buffer and processed for measurement of luciferase activity, as described above.

3.3.5.3 Haemolysis

Guinea pig erythrocytes were washed three times with PBS (5 min, 2000 rpm), and resuspended in PBS (1% vol/vol). For the haemolysis experiments, purified SeV ($5x10^8$ ffu) was incubated at 33°C for 45 min with 0.5 ml washed guinea pig erythrocytes. Haemolysis was measured by determining the optical density of the erythrocyte supernatant at 520 nm.

4. RESULTS

4. RESULTS

4.1 Comparison of the fusion requirements for Sendai virus and respiratory syncytial virus glycoproteins

4.1.1 Cloning and expression of Sendai virus and respiratory syncytial virus glycoproteins

The gene encoding the RSV fusion protein (F_RSV) has been previously subcloned into the pTM1 expression vector (González-Reyes *et al*., 2001). The genes encoding the SeV fusion (F_SeV) and attachment (HN) proteins were thus subcloned from the pGEM-4 expression vector into pTM1. The pTM1 vector (Fig. 4.1A) permits expression of the cloned gene of interest under transcriptional control of the T7 bacteriophage promoter (Elroy-Stein *et al*., 1989). In addition, pTM1 contains the encephalomyocarditis virus (ECMV) untranslated region (5' UTR), situated immediately downstream of the T7 promoter. The ECMV UTR results in increased efficiency of translation of T7-driven transcripts by a cap-independent mechanism, resulting in increased expression of the cloned gene of interest (Moss *et al*., 1990). Subcloning of SeV F and HN genes was carried out as described in the Materials and Methods. Expression of SeV pTM1-F and pTM1-HN plasmids (Fig. 4.1B) was confirmed by immunofluoresence of transfected BSR-T7/5 cells (a BHK-derived cell line that constitutively express T7 RNA polymerase) at 24 h post-transfection, using antibodies directed against the F protein (monoclonal antibody GB5), or HN protein (rabbit polyserum HN_SeV_CT).

Similarly, the gene encoding the RSV attachment (G) glycoprotein was subcloned into pTM1. However, as detailed in the Materials and Methods, it was necessary to lower the temperatures at which competent bacteria were grown. Expression of pTM1-G (Fig. 4.1B) was subsequently confirmed by immunofluoresence of transfected BSR-T7/5 cells at 48 h post-transfection, using monoclonal antibody (63G) against the G protein (Garcia-Barreno *et al*., 1992).

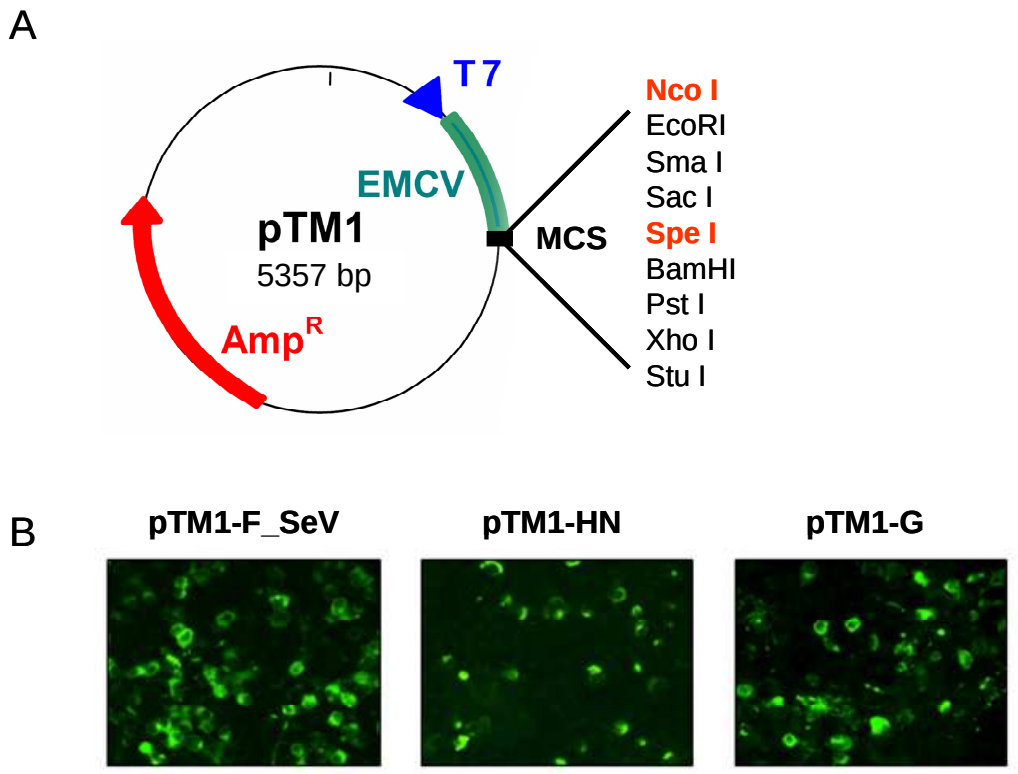


Figure 4.1. Cloning and expression of Sendai virus and respiratory syncytial virus glycoproteins.

(A) Schematic diagram of the pTM1 plasmid. The T7 promoter is shown as a blue arrow and the ampicillin resistance gene (Amp^R) as a red arrow. Also indicated is the ECMV UTR (green), and the multiple cloning site (MCS), with restriction enzymes used for subcloning of the paramyxovirus genes highlighted in red. (B) Expression of cloned glycoproteins in transfected BSR-T7/5 cells was detected using monoclonal antibodies against the SeV F protein (GB5), RSV G protein (63G), or a polyclonal rabbit serum raised against the SeV HN protein (HN_SeV_CT).

4.1.2 Comparison of the requirements of respiratory syncytial virus and Sendai virus fusion proteins for cell-cell fusion

It has previously been reported that RSV F protein (F_RSV) is able to fuse cells when expressed in transfected cells in the absence of the attachment G protein (González-Reyes *et al*., 2001; Zimmer *et al*., 2001a). In contrast, SeV F protein (F_SeV) requires both trypsin and co-expression of the attachment HN protein in order to fuse cell membranes (Tanabayashi and Compans 1996). Thus, we aimed to confirm the distinct requirements for cell-cell fusion of RSV and SeV F proteins. BSR-T7/5 cells were transfected with pTM1 plasmids containing RSV

or SeV glycoproteins, and incubated in the absence or presence of 0.25 µg/ml trypsin for 32 h (F_SeV), or 48 h (F_RSV). Fixed BSR-T7/5 cells were subsequently immunostained using monoclonal antibodies directed against F_SeV or F_RSV proteins (Fig. 4.2A).

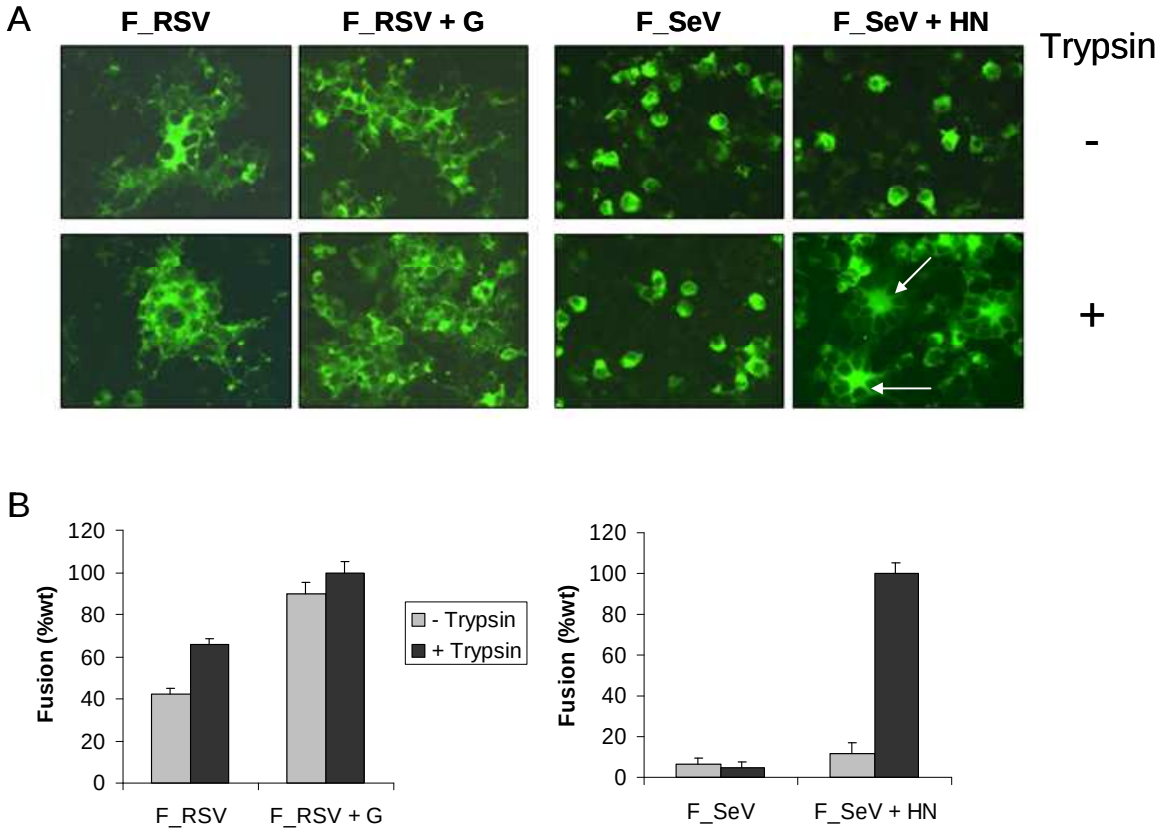


Figure 4.2. RSV and SeV fusion proteins have different requirements for cell-cell fusion. (A) BSR-T7/5 cells were transfected in microchamber culture slides with pTM1 plasmids pTM1-F_RSV or pTM1-F_SeV and pTM1-G or pTM1-HN plasmids, as indicated. The transfection mixture was removed 7 h post-transfection and cells were incubated in serum-free medium with (+) or without (-) trypsin. Cells were fixed 32 h post transfection (SeV) or 48 h post transfection (RSV), and immunostained as indicated in the Materials and Methods. Syncytia are indicated by arrows for F_SeV. (B) BSR-T7/5 cells in 48-well plates were transfected with plasmids encoding RSV or SeV genes. BHK cells were simultaneously transfected with a plasmid encoding the luciferase gene (pTM1-Luc). Seven hours post-transfection, BHK cells were detached, resuspended at a density of $1x10^6$ cells/ml in serum-free medium, and overlaid at a 1:1 ratio onto BSR cells, either in the presence or absence of trypsin. At 30 h post-transfection (or 48 h post transfection for experiments involving F_RSV), cells were lysed and analysed for luciferase activity. Results (relative light units) are expressed as a percentage of wild-type (wt) fusion (F_SeV + HN + trypsin or F_RSV + G + trypsin). Mean values from three independent experiments are shown.

Formation of syncytia (multinuclear, fused cells) was observed in transfected cells that expressed F_RSV as the only viral protein, both in the absence and presence of trypsin. The number and size of syncytia was enhanced by co-expression of F_RSV with the attachment G protein, and by the presence of trypsin in the culture medium. In contrast, F_SeV displayed a strict requirement for both HN co-expression and trypsin for syncytia formation.

The results of the syncytium formation assay were confirmed by a quantitative, luciferase reporter gene fusion assay (Fig. 4.2B). BSR-T7/5 cells transfected with the viral genes of interest were mixed with BHK cells, which had been previously transfected with the pTM1-Luc plasmid, and left to fuse in the absence or presence of trypsin. Since pTM1-Luc contains the luciferase reporter gene under transcriptional control of the T7 promoter, expression of luciferase only takes place in BHK cells that have fused with BSR-T7/5 cells (that constitutively express T7 RNA polymerase), thus permitting quantitative measurement of cell-cell fusion. While trypsin produced only a moderate enhancement in the level of fusion induced by F_RSV, co-expression of G increased cell-cell fusion by approximately two-fold. Therefore, while not a strict requirement, G enhances F_RSV-mediated cell-cell fusion. In contrast, and in accordance with the syncytium formation assay, F_SeV required both HN co-expression and trypsin in order to produce significant cell-cell fusion.

4.2 Generation and expression of Sendai virus fusion protein cleavage site mutants

4.2.1 Construction of Sendai virus fusion protein cleavage site mutants

In addition to their distinct requirements for cell-cell fusion, a comparison of the primary structure of RSV and SeV F proteins reveals some key differences. SeV F protein contains a single arginine residue at its cleavage site (R116), thereby necessitating the addition of trypsin to cell culture medium for F protein cleavage. In contrast, RSV F possesses two furin-consensus cleavage sites (site I RARR109 and site II KKRKR136), which are separated by a region of 27 amino acids (pep27). Alignment of F_RSV and F_SeV primary sequences (Fig.

4.3) reveals that although both proteins have homologous fusion peptides, the two multibasic cleavage sites and the intervening segment of F_RSV cannot be aligned with F_SeV, resulting in a gap in the alignment.

Figure 4.3. Schematic diagram of SeV F protein cleavage site mutants. Alignment of F_RSV (amino acids 100-144, red) and F_SeV (amino acids 99-129, blue), with gaps in the alignment indicated by dashed lines. Cleavage sites of F_RSV (site I, RARR109 and site II, KKRKRR136) and F_SeV (R116) are shown in bold and indicated by arrows. The fusion peptide is located immediately proceeding the cleavage site. Residues from F_RSV that were inserted into a backbone of F_SeV are shown in red.

Thus, we aimed to investigate if the unique characteristics of the F_RSV proteolytic processing region play a role in the distinct fusion requirements by F_RSV and F_SeV. Site-directed mutagenesis was carried out on the pTM1-F_SeV plasmid in order to reproduce partially, or totally, the sequences that determine cleavage of F_RSV. In an initial round of mutagenesis, residues from the second cleavage site of F_RSV were inserted directly preceding the single cleavage site of F_SeV (R116), producing F_RKR and F_KKRKR mutants, containing a minimal furin recognition sequence (RKRR), or the complete F_RSV cleavage site II (KKRKRR), respectively (Fig. 4.3). The F_KKRKR mutant was termed F_cleavage (Fc), and was subjected to further mutation in order to introduce F_RSV cleavage site I, producing the F110 mutant. As detailed in Fig. 4.3, the complete intervening sequence between the two RSV F cleavage sites (pep27) was then inserted into the F110 mutant in three stages. F117 contains both F_RSV cleavage sites I and II, separated by 27 intervening amino acids derived from both F_RSV and F_SeV. FΔ was subsequently produced from the F117 mutant by a deletion N-terminal to the second cleavage site, resulting in a shortened intervening segment, composed entirely of F_RSV residues. Finally, the remaining residues of F_RSV pep27 were inserted into FΔ to produce the F130 mutant, which contains the complete F_RSV pep27 sequence.

4.2.2 Expression of Sendai virus fusion protein cleavage site mutants in transfected cells

The relative cell surface expression levels (in comparison to wild-type F_SeV) of the distinct F_SeV cleavage mutants was determined by flow cytometry of transfected BSR-T7/5 cells, using the monoclonal antibody GB5 directed against F_SeV. All F_SeV cleavage site mutants were expressed at the cell surface at levels comparable to the wild-type (wt) F_SeV protein (Fig. 4.4A), although a slight decrease in expression was noted for F_SeV mutants containing both F_RSV cleavage sites. Expression was also analysed by immunoprecipitation of transfected cell extracts in order to compare proteolytic cleavage of the mutant SeV F proteins. Monoclonal antibody GB5 was purified by affinity chromatography and conjugated to Sepharose 4B beads, which were

used to immunoprecipitate SeV F proteins from membrane extracts of transfected BSR-T7/5 cells. The resulting immunoprecipitates were separated by SDS-PAGE, and visualised by Western blot using biotinylated GB5 antibody and a streptavidin-PO conjugate.

In the absence of trypsin, both F_SeV wild-type and the single cleavage site mutant F_RKR remained essentially uncleaved, as only a faint band representing cleaved F1 was observed (Fig. 4.4B, upper panel). In contrast, the Fc mutant and double cleavage site mutants F110, F117 and F130 were cleaved to a greater extent in the absence of trypsin. A band migrating at a lower molecular weight than F0 was observed for the mutant FΔ. Although the identity of this band has not been thoroughly investigated, it may represent the F0 precursor of the FΔ mutant, which lacks an N-glycosylation site that has previously been shown to be used in the Z strain of SeV F protein (N104), (Segawa *et al*., 2000). On the addition of trypsin to the culture medium of transfected cells (Fig. 4.4B, lower panel), cleavage of all F proteins was enhanced. In particular, double cleavage site F_SeV mutants were almost completely cleaved to F1, as only a faint band corresponding to uncleaved F0 was seen. Thus, insertion of F_RSV cleavage site II in SeV F protein increases the extent of cleavage at this site in the absence of trypsin. The susceptibility of site II to trypsin cleavage is further enhanced by insertion of F_RSV cleavage site I upstream of site II, as observed for the double cleavage site mutants.

It has previously been shown that in the absence of cleavage at site II, cleavage of F_RSV at site I results in a partially-cleaved protein (F1+), seen as an intermediate band between F0 and F1 (González-Reyes *et al*., 2001). However, F1+ was not observed for the F_SeV double cleavage site mutants, suggesting that site I is either not cleaved, or that if cleavage at site I does take place, it is immediately proceeded by cleavage at site II to form F1. Similarly, König *et al*., (2004) also failed to observe the presence of F1+ in transfected cells, suggesting that the ability to observe this partially-cleaved intermediate is dependent on cell type and/or expression system.

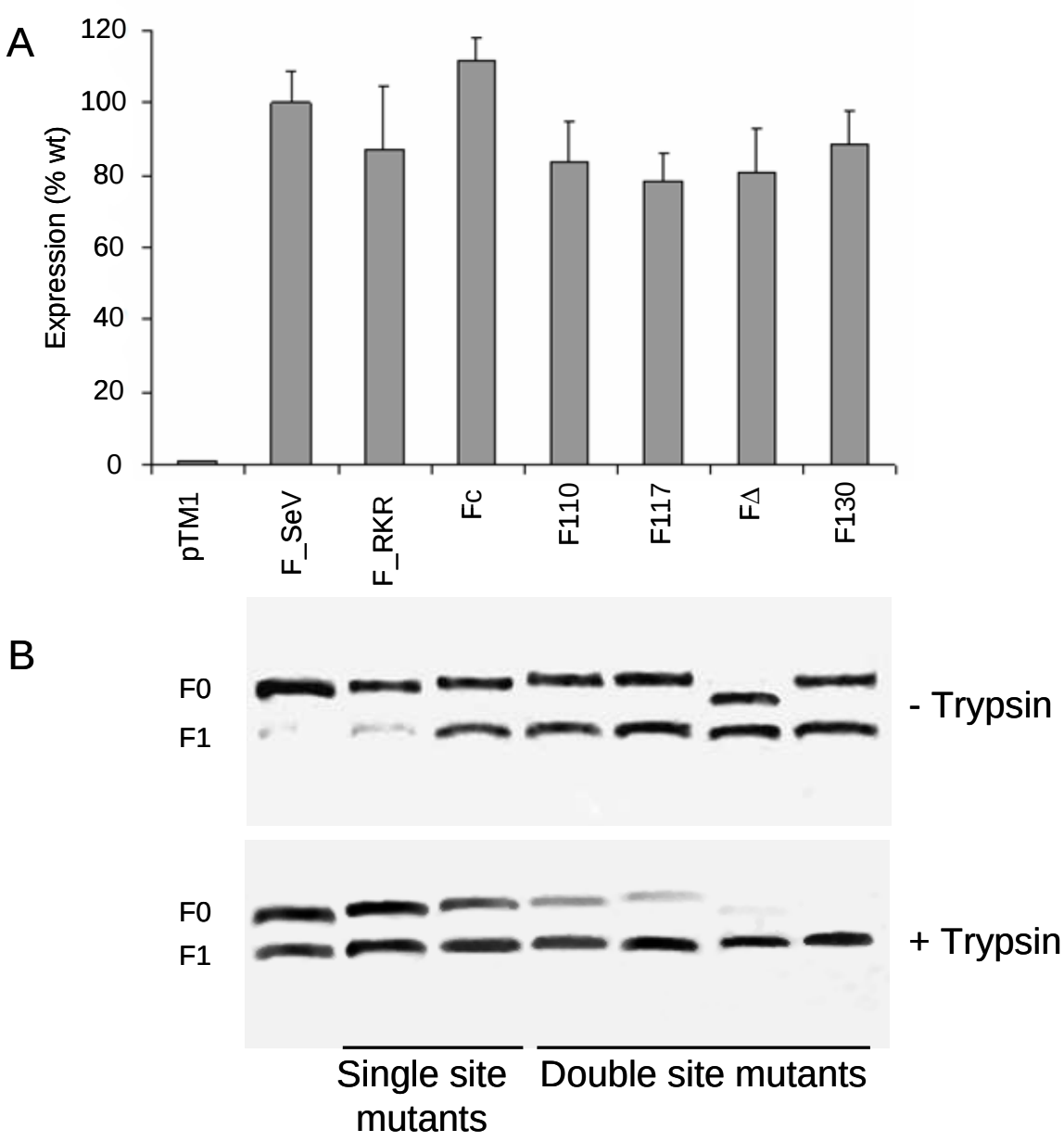


Figure 4.4. Cell surface expression and proteolytic cleavage of chimeric SeV F mutants. (A) Cell surface expression levels of wild-type and mutant F_SeV proteins were determined by flow cytometry analysis of transfected BSR-T7/5 cells 24 h post-transfection, using the monoclonal antibody GB5. The mean fluorescent intensity of cells is represented as a percentage of wild-type F_SeV expression, and mean values from three independent experiments are shown. (B) Extracts of membrane proteins were prepared from transfected BSR-T7/5 cells incubated in the absence (upper panel) or presence (lower panel) of trypsin, and subjected to immunoprecipitation using purified GB5 antibody bound to Sepharose beads. The immunoprecipitated F_SeV proteins were subsequently fractionated by SDS-PAGE under reducing conditions, and analysed by Western blotting using biotinylated GB5 antibody and a streptavidin-horseradish peroxidase conjugate.

4.3 Effect of cleavage site mutants on the ability of paramyxovirus fusion proteins to direct cell-cell fusion

4.3.1 Formation of syncytia in the absence or presence of the HN attachment protein by Sendai virus fusion protein cleavage site mutants

Syncytium formation by F_SeV cleavage mutants was tested by transfection of BSR/T7-5 cells with F_SeV mutants in the absence or presence of HN co-expression. In the presence of the HN attachment protein, only small syncytia were observed for F_RKR when trypsin was excluded from the culture medium, as indicated by the arrows in Fig. 4.5A, reflecting the poor cleavage of F_RKR in the absence of trypsin (Fig. 4.4B). In contrast, Fc formed large syncytia in the absence of trypsin (Fig. 4.5A), consistent with the enhanced cleavage of the Fc mutant (Fig. 4.4B). However, although Fc was capable of forming more extensive syncytia than wt F_SeV, (compare with Fig. 4.2), both single cleavage site F_RKR and Fc mutants continued to require HN co-expression in order to form syncytia, since only individual, unfused cells were observed in the absence of HN co-expression (Fig. 4.5B). Double cleavage site F_SeV mutants formed extremely large syncytia in the presence of HN, without a requirement for trypsin (Fig. 4.5A). Moreover, and in contrast to single cleavage site mutants, all double cleavage site F_SeV mutants were also able to form syncytia in the absence of HN co-expression (Fig. 4.5B). Therefore, inclusion of the two multibasic F_RSV cleavage sites in F_SeV facilitates syncytium formation in the absence of an attachment protein, thus mimicking the unique properties of F_RSV. Although HN-independent cell-cell fusion was observed in the absence of trypsin, trypsin inclusion did enhance the size and number of syncytia formed. The ability of the F110 mutant to form syncytia in the absence of HN implies that insertion of both F_RSV cleavage sites and not the intervening pep27 sequence *per se* conveys HN-independence for cell-cell fusion. However, the sequence and/or length of the intervening region between the two cleavage site does appear to influence the extent of fusion, since F117 forms larger syncytia than FΔ in the absence of HN (Fig. 4.5B).

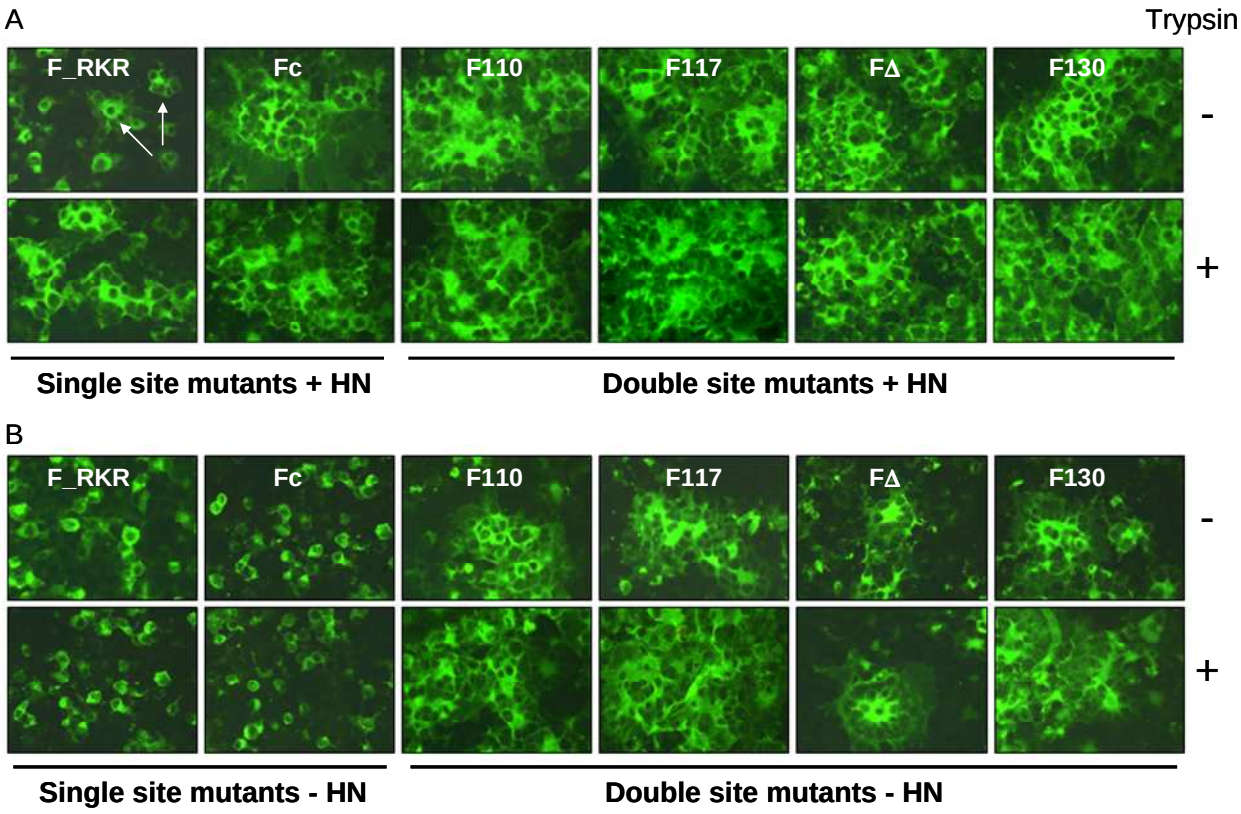


Figure 4.5. Syncytium formation by Sendai virus F protein cleavage site mutants. BSR-T7/5 cells growing in microchamber wells were transfected with pTM1 plasmids encoding wt or mutant F_SeV genes with (A) or without (B) pTM1-HN. Transfected cells were processed for syncytia formation 32 h post-transfection, as described in the legend to Fig. 4.2A.

4.3.2 Cell content mixing in the absence or presence of the HN attachment protein by Sendai virus fusion protein cleavage site mutants

In order to confirm the results of the syncytium formation assay, all of the mutants detailed in Fig. 4.3 were analysed for cell content mixing using the luciferase reporter gene assay (Fig. 4.6). Luciferase activity was expressed as a percentage of fusion relative to wt F_SeV co-expressed with HN in the presence of trypsin, which represents 100% fusion. As shown in Fig. 4.6A, the F_RKR mutant produced only 50% of wt fusion in the absence of trypsin, and fused cells to the same extent as the wt F protein on addition of trypsin. In contrast, Fc produced 181% of wild-type F_SeV fusion in the absence of trypsin. Thus, the presence of six basic residues at the cleavage site of F_SeV enhances the fusogenic potential of this protein when co-expressed with HN, in the absence or presence of trypsin. In the presence of HN, all double cleavage

site F_SeV mutants displayed dramatically increased fusion with respect to the wt F protein (between three- and four-fold in the presence of trypsin), with the intervening sequence between the two cleavage sites exerting a minor, modulatory role on fusion.

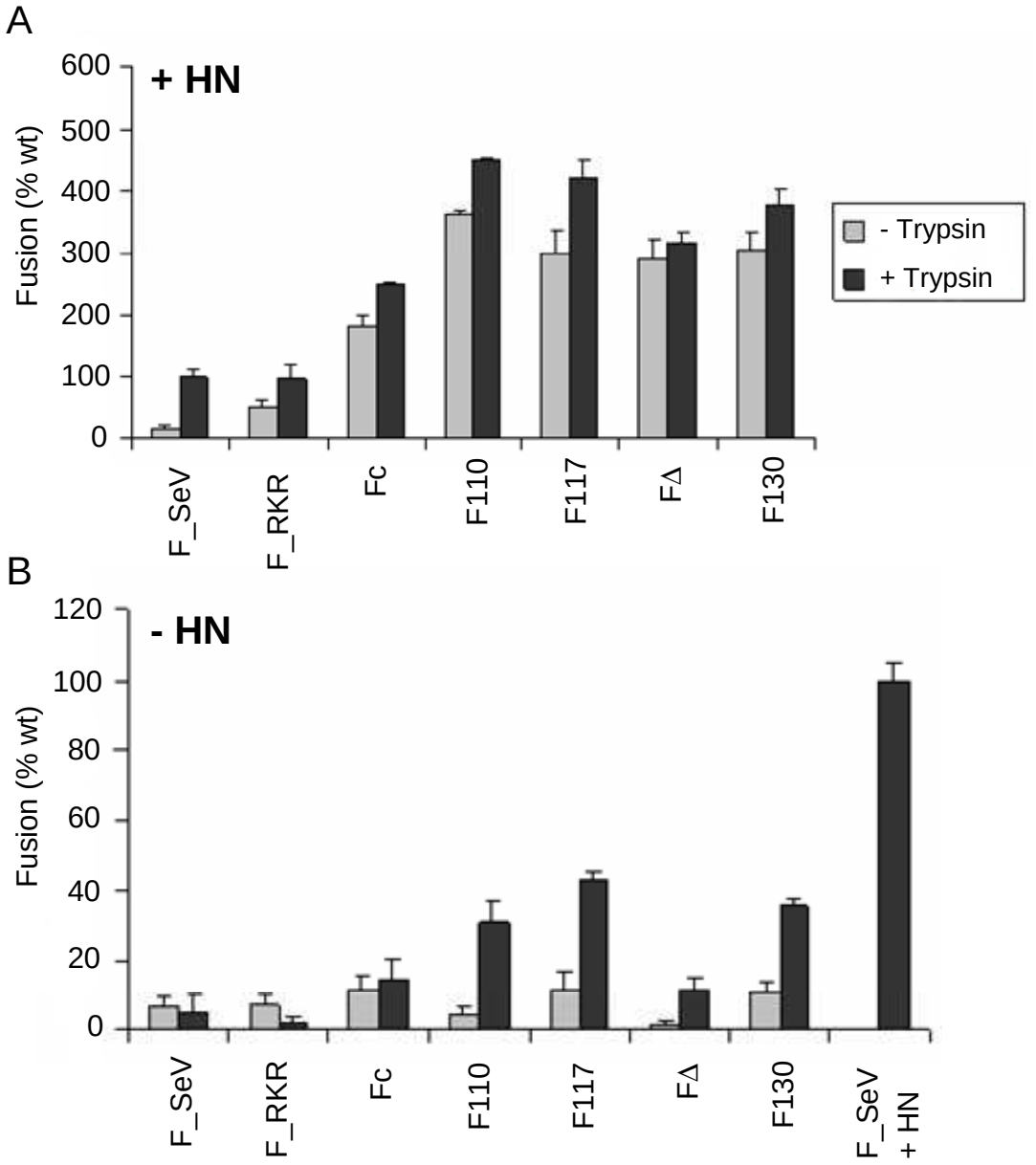


Figure 4.6. Cell-cell fusion by Sendai virus F protein cleavage site mutants. BSR-T7/5 cells were transfected with pTM1 plasmids encoding wt or mutant F_SeV genes with (A) or without (B) pTM1-HN, as indicated. Cells were mixed with BHK cells, which had previously been transfected with pTM1-Luc, either in the presence or absence of trypsin, and incubated for 22 h to allow fusion. Cells were subsequently lysed and analysed for luciferase activity at 30 h post-transfection. Results (relative light units), are expressed as a percentage of wt fusion (F_SeV + HN + trypsin), with mean values from three independent experiments shown.

In the absence of HN co-expression (Fig. 4.6B), three double cleavage site mutants (F110, F117 and F130) produced fusion activity in the luciferase reporter gene assay in the presence of trypsin. As observed in the syncytium formation assay, the single cleavage site Fc mutant did not produce significant fusion in the absence of HN. The FΔ double cleavage site mutant also failed to produce HN-independent fusion in the reporter gene assay, reflecting the small syncytia produced by this mutant in the absence of an attachment protein (Fig. 4.5B).

The enhanced fusogenicity of double cleavage site mutants may reflect their increased susceptibility to cleavage in the presence of trypsin (Fig. 4.4B, lower panel). However, no strict correlation between F0 processing and membrane fusion activity was observed. For instance, while FΔ was highly susceptible to trypsin cleavage, this mutant was poorly active in membrane fusion assays. Furthermore, although F130 was cleaved by trypsin more efficiently than F110 or F117 mutants (Fig. 4.4B, lower panel), it did not produce larger syncytia or induce greater luciferase activity in the absence of HN (Figures 4.5 and 4.6).

Lower levels of HN-independent fusion were observed for the double cleavage site mutants in the luciferase reporter gene assay compared to the syncytium formation assay. This may reflect differences in the requirements of the two assays. For instance, the presence of F protein in both target and donor cells in the syncytium formation assay may facilitate cell-cell fusion with respect to the reporter gene assay, in which F protein is present only in the donor BSR-T7/5 cells. Furthermore, the reporter gene assay involves the interaction between two distinct populations of cells, and may depend to a greater extent on the attachment ability of the mutated F proteins.

4.3.3 Cell content mixing in the absence or presence of the G attachment protein by respiratory syncytial virus fusion protein cleavage site mutants

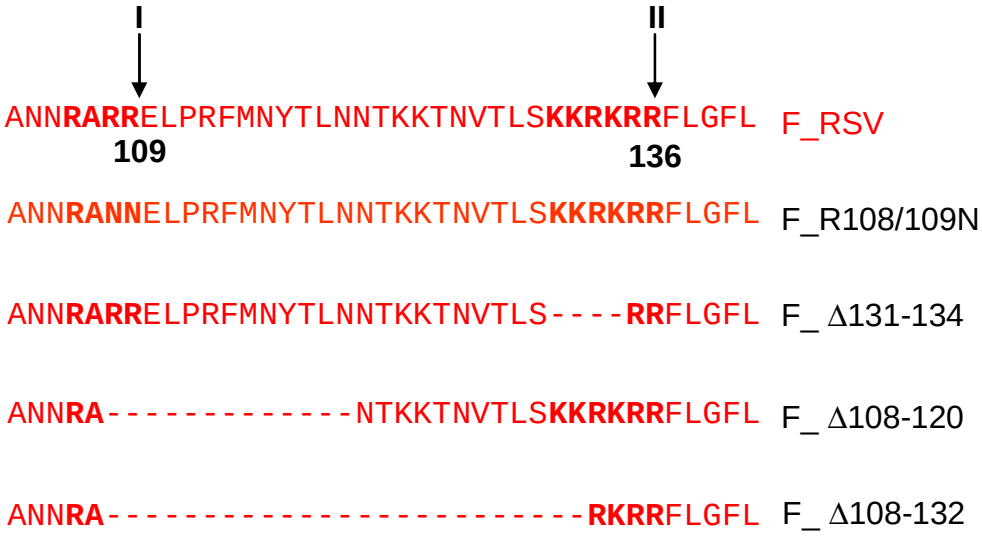
It has previously been shown that cleavage at both sites I and II is a requirement for fusion of the RSV F protein in the absence of the attachment G protein. Mutations of F_RSV cleavage site I abrogated syncytia formation in the absence of trypsin, whereas deletion of F_RSV cleavage site II prevented syncytia formation, even in the presence of trypsin (González Reyes *et al*., 2001; Zimmer *et al*., 2001a). In light of our findings that insertion of F_RSV cleavage site I in F_SeV led to syncytia formation by F_SeV in the absence of HN, we aimed to determine whether co-expression of RSV G protein with F_RSV cleavage site mutants would restore cell-cell fusion.

A series of F_RSV cleavage site mutants (Fig. 4.7A) have been previously described (González Reyes *et al*., 2001; Ruiz-Argüello *et al*., 2002; 2004). These mutations included changes in cleavage site I (RARR) to produce the F_R108/109N mutant (RANN), or deletion of four basic residues of cleavage site II (KKRKRR), resulting in the F_Δ131-134 mutant (González Reyes *et al*., 2001). Deletion of part or all of the intervening pep27 sequence of F_RSV resulted in the RSV F_Δ108-120 and F_Δ108-132 mutants, respectively (Ruiz-Argüello *et al*., 2002; 2004). All mutant proteins were expressed at the cell surface at levels comparable to F_RSV, as determined by flow cytometry of transfected cells (Fig. 4.7B), with the exception of the cleavage site II mutant F_Δ131-134, which was expressed at a reduced level (60% of wild-type F_RSV). This is consistent with previous findings that mutation of F_RSV cleavage site II increases the susceptibility of the protein to degradation, suggesting that mutation of site II affects the conformation of F_RSV (Zimmer *et al*., 2001a).

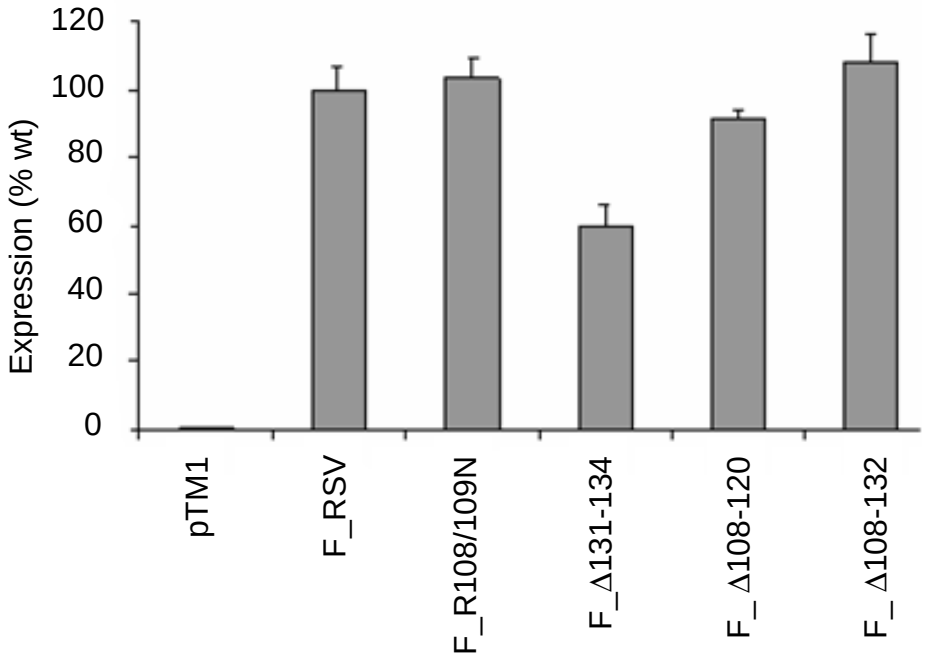
The cell-cell fusion activity of F_RSV cleavage mutants was analysed by the luciferase fusion assay (Figures 4.7C and 4.7D). Cleavage site I mutant F_R108/109N produced a low level of fusion, which was increased by the presence of trypsin, but not by co-expression of the G protein. Cleavage site II

mutant F_Δ131-134 failed to produce fusion above background levels, irrespective of the inclusion of trypsin or G protein. Further mutation of F_RSV by deletion of pep27 to produce F_Δ108-120 and F_Δ108-132 mutants led to an increase in cell-cell fusion activity compared to F_R108/109N, in particular in the presence of the attachment G protein. However, the fusion activity of both F_Δ108-120 and F_Δ108-132 in the presence of G was approximately half that of the wild-type, (54% and 49% of wt F co-expressed with G in the presence of trypsin, respectively). Therefore, co-expression of the G protein failed to fully rescue the reduced fusogenic capacity of the RSV F cleavage site mutants.

A



B



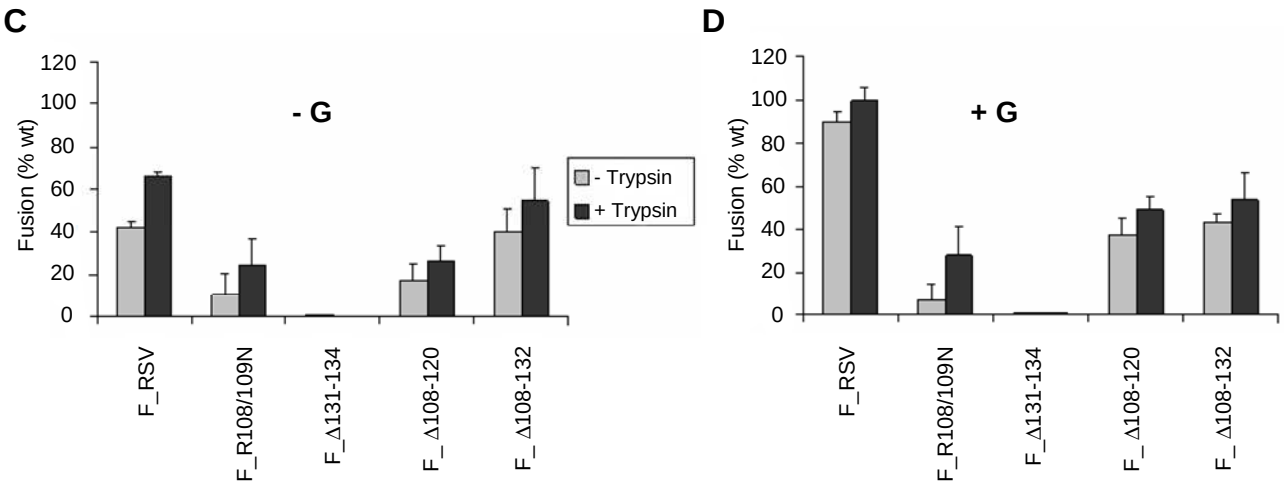


Figure 4.7. Cell-cell fusion by RSV F cleavage site mutants. (A) The amino acid sequence surrounding the F_RSV proteolytic processing region (amino acids 103 to 141) is shown. F_RSV cleavage sites (site I, RARR109 and site II, KKRKRR136) are shown in bold and indicated by arrows. Deleted residues are shown by a dashed line. (B) The level of cell surface expression of wild-type (wt) and mutant F_RSV proteins was determined by flow cytometry of transfected BSR-T7/5 cells 24 h post-transfection, using the monoclonal antibody 2F. The mean fluorescent intensity of cells is represented as a percentage of wt F_RSV expression, with mean values from three independent experiments shown. (C) BSR-T7/5 cells were cotransfected in 48-well plates with pTM1 plasmids encoding wt or mutant F_RSV genes in the absence (-G) or presence (+G) of pTM1-G. BHK cells were simultaneously transfected with pTM1-Luc and overlaid at a 1:1 ratio onto BSR-T7/5 cells. Cells were lysed and analysed for luciferase activity at 48 h posttransfection. Results (relative light units) are expressed as a percentage of wt fusion (F_RSV + G + trypsin), with mean values from three independent experiments shown.

4.4 Generation and expression of recombinant Sendai viruses containing fusion protein cleavage site mutations

4.4.1 Subcloning of Sendai virus fusion protein cleavage site mutants in the full length Sendai virus cDNA plasmid

Given the previously described differences between paramyxovirus cell-cell and virus-cell fusion (Connolly and Lamb 2007), it was of interest to analyse the effect of F_SeV cleavage site mutations on fusion and infection by Sendai virus. To this aim, recombinant Sendai virus (rSeV) was generated from a cDNA clone (FL5). As shown in Fig. 4.8A, FL5 is an 18.3 kb plasmid that contains the full length SeV cDNA (Z strain) and various unique restriction enzyme sites. The genes encoding wild-type SeV F from the Harris strain (FH), and SeV cleavage mutants Fc, F110, F117 and F130 (see Fig. 4.9A), were subcloned from pTM1 into the FL5 plasmid between the Mlu I and Rsr II restriction sites, replacing the wild-type SeV F gene (Z strain) present in FL5. (see section 3.3.3.1 and Table 3.1 of the Materials and Methods for details of the subcloning protocol and oligonucleotides employed).

4.4.2 Rescue of recombinant Sendai virus containing mutations at the fusion protein cleavage site

The minimal infectious unit of negative-strand RNA viruses is the ribonucleoprotein complex (RNP), which comprises nuclear (N), phosphoprotein (P), and large RNA-dependent RNA polymerase (L) proteins. Thus, in order to rescue rSeV, BSR-T7/5 cells that constitutively express T7 polymerase were co-transfected with FL5 cDNA and pTM1 plasmids carrying the N, P and L genes under transcriptional control of the T7 promoter (Fig. 4.8B). Transcription of transfected plasmids results in the production of antigenomic (+) RNP, thus permitting the assembly of genomic (-) RNP after one successful round of replication driven by the plasmid-encoded support proteins (for a review, see Conzelmann 1998).

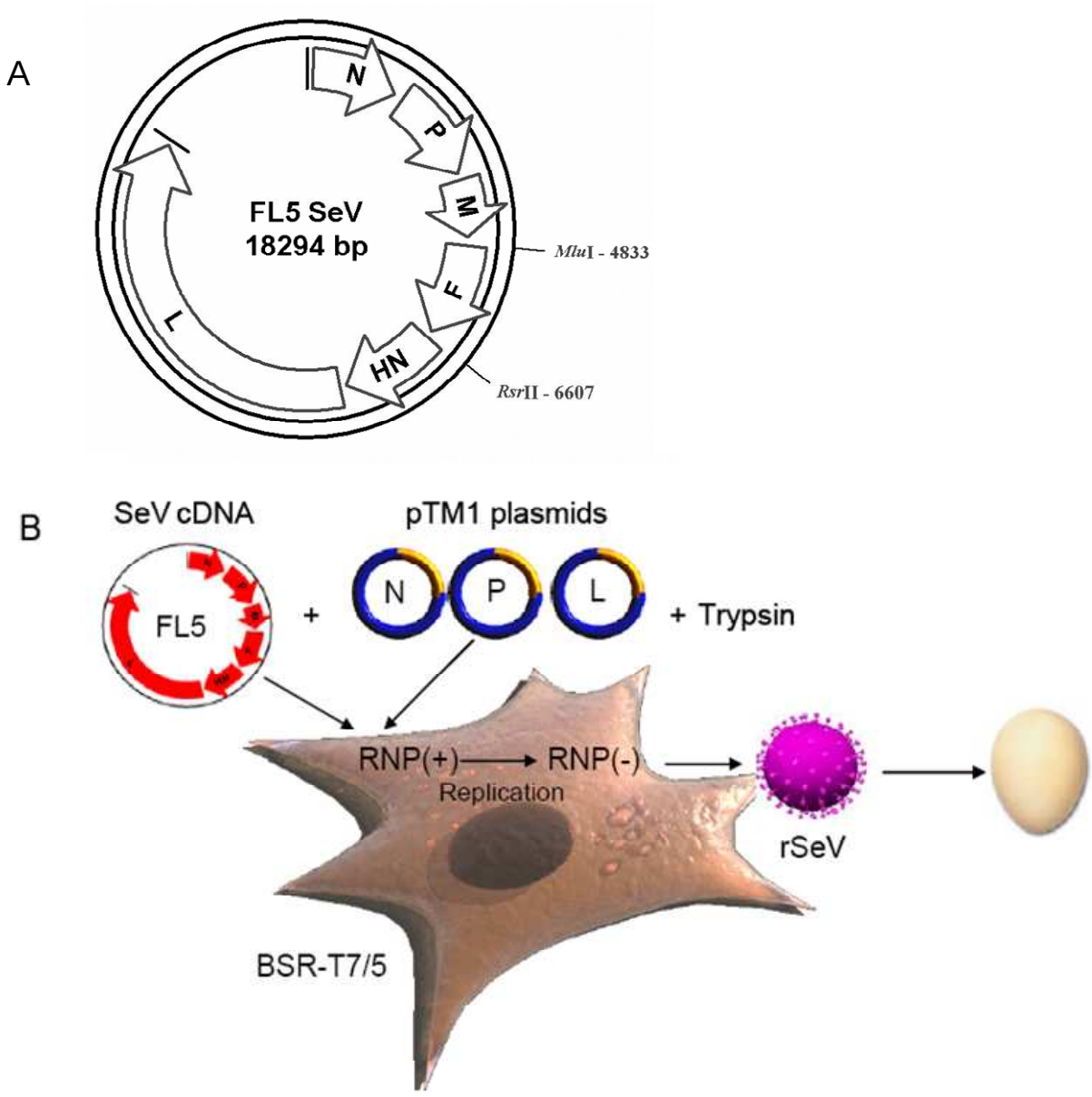


Figure 4.8. Outline of the rescue of recombinant Sendai virus. (A) The plasmid (FL5) encoding the full length cDNA Sendai virus genome is shown (drawn using pDRAW32 1.1.105 DNA analysis software). Sendai virus genes are indicated by arrows, and unique restriction sites Mlu I and Rsr II employed in the subcloning of F_SeV genes are shown. (B) In order to rescue recombinant SeV from the FL5 cDNA clone, BSR-T7/5 cells that constitutively express T7 polymerase were co-transfected with FL5 and pTM1 plasmids carrying the N, P and L genes under transcriptional control of the T7 promoter. Cells were harvested 72 h post transfection and injected into 9-day-old embryonated chicken eggs, which permit the growth of SeV to high titres (Garcin *et al.*, 1995). RNP; ribonucleoprotein complex.

Transfected BSR-T7/5 cells were incubated in the absence or presence of trypsin at 33℃ for 72 h and the cell culture supernatant was subsequently passaged three times in 9-day-old embryonated chicken eggs to increase viral yield (Garcin *et al*., 1995). In order to detect the presence of viral proteins, allantoic fluid was pelleted through a 25% glycerol cushion for 30 min at 15,000 rpm and loaded onto a 10% SDS-PAGE gel. As seen in Fig. 4.9B, all rSeV (SeV-FH, SeV-Fc, SeV-F117 and SeV-F130) were successfully rescued. However, titration of the allantoic fluid by the method of Borisevich *et al*., (2008), revealed that double cleavage site mutants SeV-F117 and SeV-F130 were attained at lower titres than single cleavage site mutants SeV-FH and SeV-Fc, as detailed in the legend of Fig. 4.9B. Whether these differences reflect lower replication or lower efficiency of rescue of the double cleavage site viruses compared with single cleavage site viruses is not known at present. The band representing the M protein appears fainter and additional bands are present for the double cleavage site mutants, in particular for F117. This may reflect degradation of purified virus on SDS-PAGE sample preparation or the presence of contaminating bands from the allantoic fluid on virus purification. Sequencing of RT-PCR products confirmed that the F and HN genes had not acquired spurious mutations during the rescue (not shown).

F protein expression by rSeV was confirmed by Western blotting with a polyclonal serum directed against the cytoplasmic tail (CT) of F_SeV (Fig. 4.9C). Faint bands migrating with a size corresponding to F0 were observed only for single cleavage site FH and Fc mutants, suggesting that almost complete cleavage of the F proteins to F1 had taken place. It is likely that trypsin-like endoproteases present in embryonated chicken eggs are responsible for cleavage of the F protein (Gotoh *et al*., 1990; Muramatsu and Homma 1980).

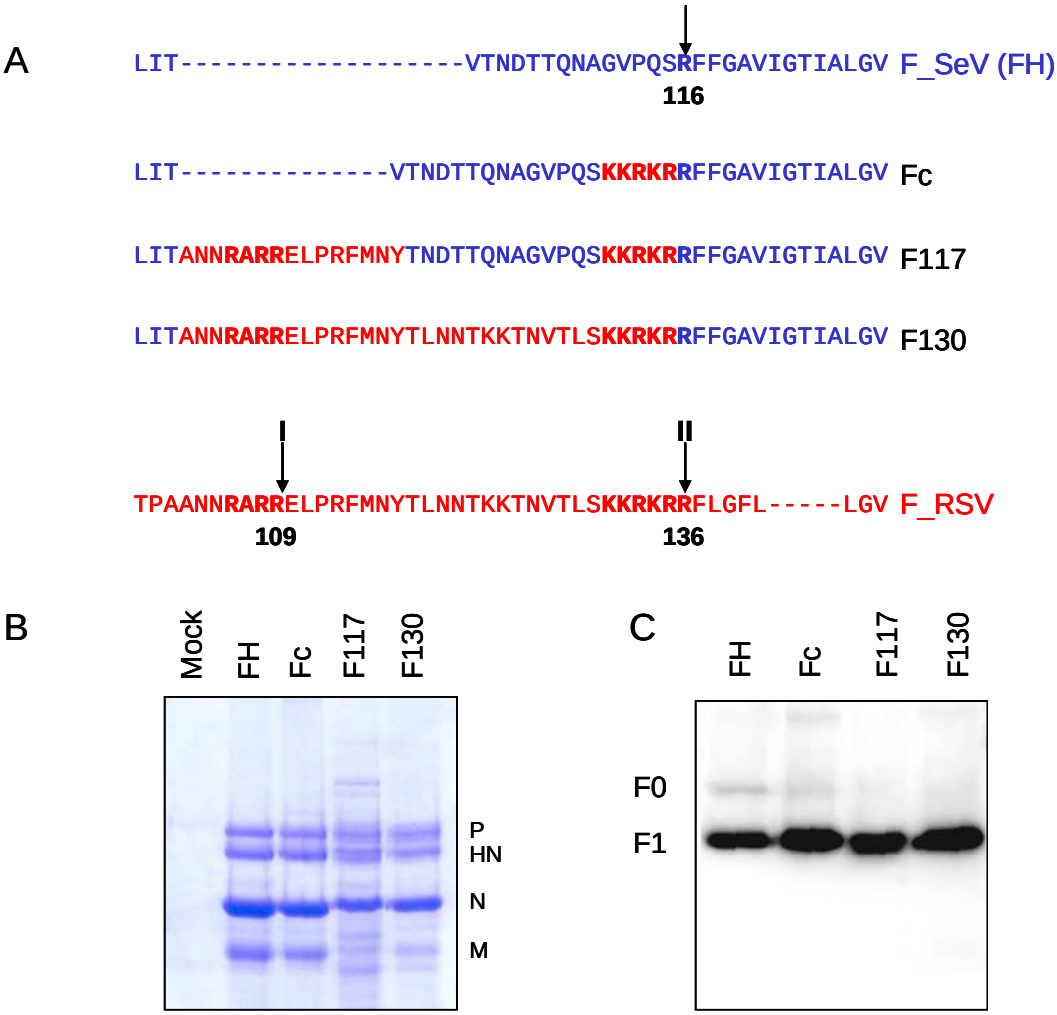


Figure 4.9. Rescue of recombinant Sendai viruses (rSeV). (A) Alignment of SeV fusion protein (F_SeV), (amino acids 99-129, blue) and RSV fusion protein (F_RSV), (amino acids 100-144, red). Cleavage sites of F_RSV (site I, RARR109 and site II, KKRKRR136) and F_SeV (R116) are shown in bold and indicated by arrows. Residues from F_RSV that were inserted into the F_SeV backbone to produce cleavage mutants are shown in red. Recombinant Sendai virus expressing the F protein mutants was rescued by co-transfection of BSR-T7/5 cells with the FL5 plasmid containing the complete SeV cDNA and pTM1 plasmids encoding SeV N, P and L genes. Rescued virus was subsequently passaged three times in embryonated chicken eggs. Titration of the allantoic fluid revealed that double cleavage site viruses were obtained at lower titres compared to single cleavage site viruses (SeV-FH: $5x10^{11}$, Fc: $9x10^{11}$, F117: $9x10^{10}$, F130: $5x10^{10}$). Allantoic fluid from the third passage in eggs was clarified, centrifuged through a 25% glycerol cushion, and equivalent infectious titres of SeV-FH, SeV-Fc, SeV-F117 and SeV-F130 were analysed on a 10% SDS-PAGE gel stained with Coomassie blue (B) or by Western blotting (C) using the polyclonal rabbit serum (F_SeV_CT), which recognises the cytoplasmic tail of F_SeV. (P, phosphoprotein; HN, haemagglutinin-neuraminidase; N, nucleoprotein; M, matrix protein; F0, uncleaved F protein precursor; F1, cleaved F protein).

4.4.3 Titration and growth of recombinant Sendai virus

In order to compare the growth of rSeV over multiple replication cycles, LLC-MK2 cells were infected with rSeV at a low multiplicity of infection (MOI) of 0.05 and incubated in the absence or presence of trypsin for 96 h. At 24 h intervals, aliquots of cell culture supernatant were taken and replaced with fresh medium, with or without trypsin. Titration of the culture supernatant was performed by immunohistochemical staining of infected LLC-MK2 cell foci in 96-well plates (Borisevich *et al*., 2008). As can be seen from Fig. 4.10, wt SeV-FH virus required the presence of trypsin in order to replicate, consistent with previously published findings that SeV requires trypsin for F protein cleavage (Scheid and Choppin 1974).

In contrast, all of the F protein cleavage mutants replicated to high titres both in the absence or presence of trypsin, although viruses grew to higher titres (approximately one logarithm) in the presence of trypsin (Fig. 4.10B). While all of the mutants reached titres comparable to wt SeV-FH at 96 h post-infection, the double cleavage site mutants displayed slightly faster replication kinetics during the first 48 h of infection. This difference in replication kinetics was further enhanced when virus was grown at 33℃, with double cleavage site mutants replicating to titres at least one logarithm higher than single cleavage site viruses during the first 48 h of infection (Table 4.1). Therefore, insertion of both F_RSV cleavage sites in SeV F protein leads to faster replication kinetics in infected cells during the first 48 h of replication, in particular at 33℃, but does not alter the overall titres obtained after 96 h.

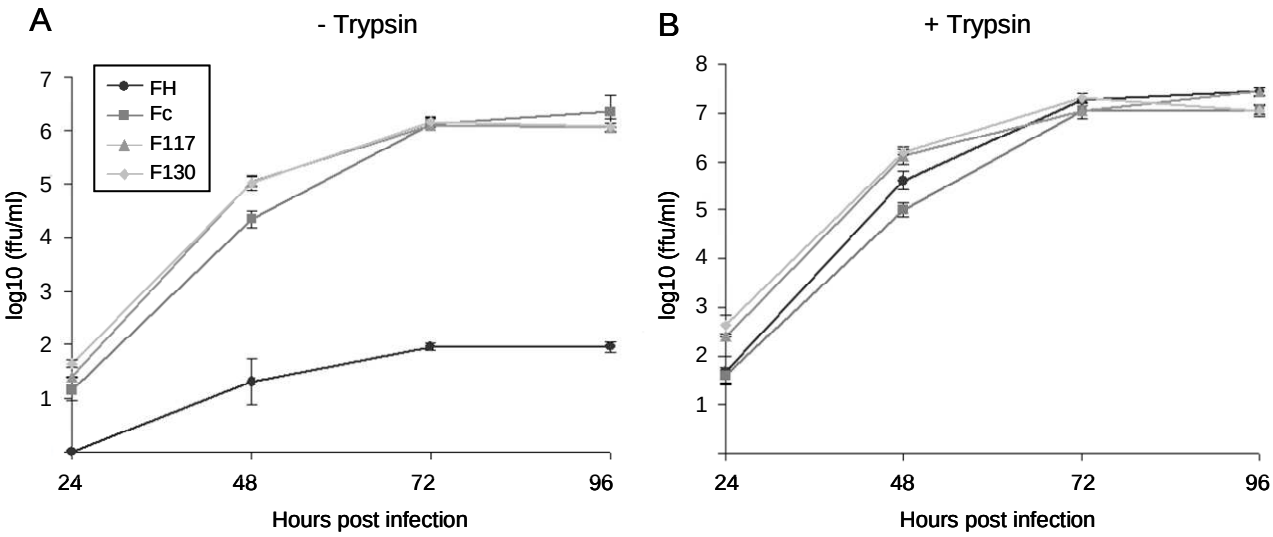


Figure 4.10. Multistep growth curve. LLC-MK2 cells were infected with an MOI of 0.05 and incubated in the absence (A) or presence (B) of trypsin for 96 hours. At 24 h intervals, aliquots of the cell culture supernatant were collected and replaced with fresh medium with or without trypsin. Titration of the culture supernatant was performed by immunohistochemical staining of infected LLC-MK2 cell foci in 96-well plates. Results represent the mean of two independent infections, which were titrated in duplicate. Ffu, focus forming units.

Table 4.1. Titration of rSeV 48 h post-infection.

	[1]Titer	
rSeV	**37ºC**	**33ºC**
FH	$4.3x10^5 \pm 1.8$	$8.9x10^3 \pm 2.5$
Fc	$1.1x10^5 \pm 0.4$	$3.3x10^3 \pm 0.9$
F117	$1.4x10^6 \pm 0.6$	$1.1x10^5 \pm 0.6$
F130	$1.5x10^6 \pm 0.2$	$1.0x10^5 \pm 0.4$

[1]Titre (ffu/ml) 48 hours post infection of culture supernatant from LLC-MK2 cells infected with rSeV at 37ºC or 33ºC in the presence of trypsin.

4.4.4 Proteolytic cleavage of recombinant Sendai virus

Proteolytic processing of the distinct F_SeV cleavage mutants was compared by infection of LLC-MK2 cells with an MOI of 0.05. Cells were incubated in the absence or presence of trypsin for 4 days and fresh trypsin was added to the cell culture medium every 24 h. Cell culture supernatant was subsequently analysed by Western blotting with a polyclonal serum (F_SeV_CT), directed against the cytoplasmic tail of F_SeV (Fig. 4.11, upper panel). A band representing wild-type FH was not observed in the absence of trypsin (Fig. 4.11, upper-left panel), reflecting the inability of SeV-FH to grow in the absence of trypsin (Fig. 4.10). The single cleavage site mutant Fc was present in uncleaved F0 and cleaved F1 forms at a ratio of 34%:66%, as determined by densitometry. In contrast, only faint bands representing the F0 precursor were seen for the double cleavage site mutants F117 and F130 in the absence of trypsin (Fig. 4.11, upper-left panel). However, and in contrast to the results seen with transfected cells (Fig. 4.4B), prominent bands representing a partially-cleaved intermediate (F1+) were observed for both F117 and F130. The partially-cleaved intermediate F1+ has previously been observed for F_RSV, and results from cleavage at site I in the absence of cleavage at site II (González-Reyes *et al*., 2001; Ruiz-Argüello *et al*., 2002). The presence of F1+ was confirmed by the use of a polyclonal serum F_RSV_104-117 (Fig. 4.11, lower-left panel), which recognises amino acids 104-117 of F_RSV, (an epitope included in the intervening pep27 region between the two cleavage sites that is also present in the F117 and F130 mutant proteins). The difference in migration between intermediate cleavage products for F117 and F130 may reflect differential glycosylation of pep27, since F117 contains 2 potential N-glycosylation sites within pep27, whereas F130 possesses 3 potential sites (see Fig. 4.9A). It has previously been estimated that at least two of the three sites are N-glycosylated for F_RSV pep27 (Zimmer *et al*, 2001a). The absence of an F1+ intermediate cleavage product in transfected cells (Fig. 4.4B) may result from the different expression system used or the distinct antibody (MAb GB5) employed in Western blotting.

Interestingly, the proportion of fully-cleaved F1 protein was approximately the same for single cleavage site mutant Fc (66%) as double cleavage site mutants F117 (64%) and F130 (66%), suggesting that the insertion of a second, upstream cleavage site does not significantly affect cleavage of site II. In the presence of trypsin, the F protein of all rSeV was completely cleaved to F1 (Fig. 4.11, upper-right panel). Completion of cleavage of F117 and F130 at both sites was additionally confirmed by loss of reactivity with the F_RSV_104-117 serum (Fig. 4.11, lower-right panel). Thus, both cleavage sites I and II are functional in double cleavage site mutants SeV-F117 and SeV-F130.

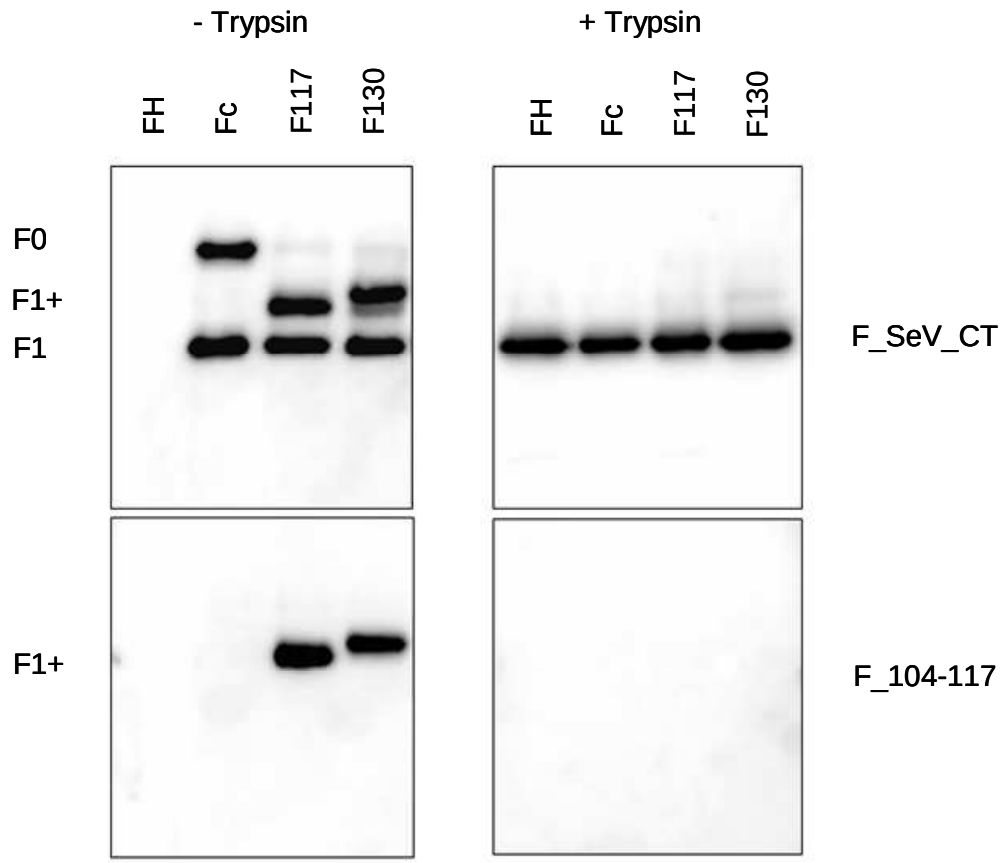


Figure 4.11. Proteolytic cleavage of rSeV. Western blotting was employed to analyse the cell culture supernatant from infected LLC-MK2 cells (MOI 0.05) incubated for 96 h in the absence (left panel) or presence (right panel) of trypsin. Western blots were developed using polyclonal rabbit sera F_SeV_CT (upper panel), which recognises SeV F protein, or F_RSV_104-117 (lower panel), which recognises amino acids 104-117 of the RSV F protein (pep27). Also indicated are bands representing uncleaved F protein precursor (F0), the fully-cleaved F1 chain (F1) and the partially-cleaved intermediate (F1+), which results from cleavage at site I in the absence of cleavage at site II. The percentage of F protein cleavage to F1 in the absence of trypsin (upper-left panel); (Fc 66%, F117 64%, F130 66%), was determined by densitometry.

4.4.5 Thermostability of recombinant Sendai virus

Given the increased virus titres seen at 33℃ (Table 4.1), it was hypothesised that double cleavage site mutants may have decreased thermostability. Recombinant Sendai viruses were purified from the allantoic fluid of embryonated eggs by sucrose gradient centrifugation and pre-incubated at 37℃ for 2, 4, or 6 h. Heat-treated rSeV were subsequently tested for their ability to mediate haemolysis of guinea pig erythrocytes. Recombinant SeV were incubated at 33℃ for 45 min with 0.5 ml guinea pig erythrocytes (1% vol/vol), and the extent of haemolysis determined by measuring optical density at 520 nm. Haemolysis results from changes in membrane permeability of erythrocytes following viral fusion, and can therefore be used as a measure of virus-cell fusion. Haemolysis directed by wt SeV-FH was significantly more resistant to heat treatment than the cleavage site mutant viruses (Fig. 4.12A), retaining approximately 40% haemolytic activity even after 6 h of heat treatment, in agreement with previously published results for the fusion of SeV with liposomes (Wharton *et al*., 2000). In contrast, all cleavage site mutants were more readily inactivated by heat treatment. In particular, haemolysis directed by the double cleavage site mutant SeV-F130, which contains the complete pep27 region of F_RSV, was reduced to approximately 20% following just 4 h incubation at 37℃. We subsequently tested the ability of heat-treated virus obtained from cell culture supernatants to infect LLC-MK2 cells. As shown in Fig. 4.12B, the titre of heat-inactivated viruses reflects the haemolysis results, since wt SeV-FH was more resistant to heat treatment than the cleavage site mutants. Following 6 h incubation at 37℃, the titre of wt SeV_FH remained approximately 2 logarithms higher than the cleavage site mutant viruses, with SeV-F130 displaying the lowest titre following heat treatment. Thus, insertion of one or both F_RSV cleavage sites in F_SeV leads to reduced thermostability, since cleavage site mutants display reduced haemolytic activity and replicate to lower titres than wt SeV-FH following heat treatment. Moreover, the double cleavage site F130 mutant that contains the complete pep27 region, and thus most closely resembles F_RSV, was the least thermostable virus.

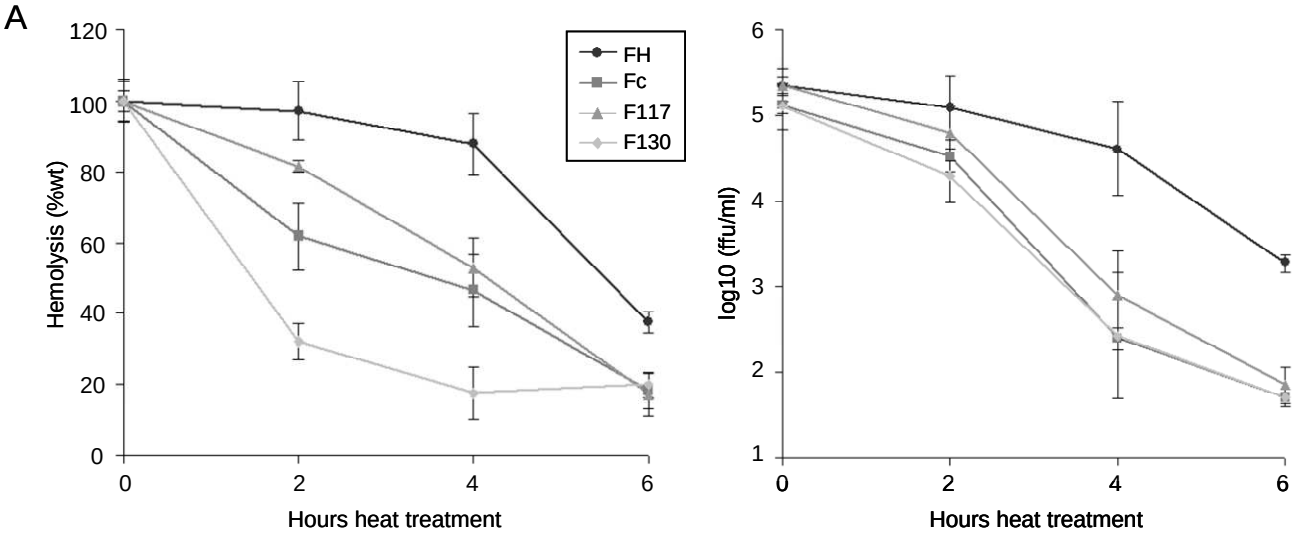


Figure 4.12. Thermostability of recombinant Sendai viruses. (A) Purified SeV was pre-incubated at 37℃ for 2, 4 or 6 hours, and subseque ntly incubated at 33℃ for 45 min with 0.5 ml washed guinea pig erythrocytes (1% vol/vol in PBS). Haemolysis was measured by determining the optical density of the erythrocyte supernatant at 520 nm. Results represent the mean of two independent experiments performed in duplicate. (B) Supernatant from infected LLC-MK2 cells was pre-incubated at 37℃ for 2, 4 or 6 hours, and subsequently titrated on LLC-MK2 cells in 96-well plates by immunohistochemical staining. Results represent the mean of three independent experiments.

4.5 Effect of fusion protein cleavage site mutations on cell-cell and virus-cell fusion by recombinant Sendai virus

4.5.1 Formation of syncytia by recombinant Sendai virus mutants

The ability of cleavage site mutant viruses to direct cell-cell fusion was compared by infection of LLC-MK2 cells grown in microchamber culture slides at an MOI of 0.5. Infected cells were incubated in the absence or presence of trypsin at 37ºC for 30 h. Fixed cells were subsequently stained with the monoclonal antibody GB5 that recognises F_SeV (Fig. 4.13A), and the extent of syncytia formation by rSeV was quantitated by counting the number of nuclei per syncytium (Fig. 4.13B). The presence of trypsin was essential for the formation of syncytia by SeV-FH, whereas all rSeV cleavage mutants formed syncytia in the absence of trypsin. As can be seen from Fig. 4.13, double

cleavage site mutant viruses formed larger syncytia than single cleavage site mutants. In particular, double cleavage site mutants SeV-F117 and SeV-F130 formed syncytia that were approximately four times the size of syncytia formed by wt SeV-FH in the presence of trypsin.

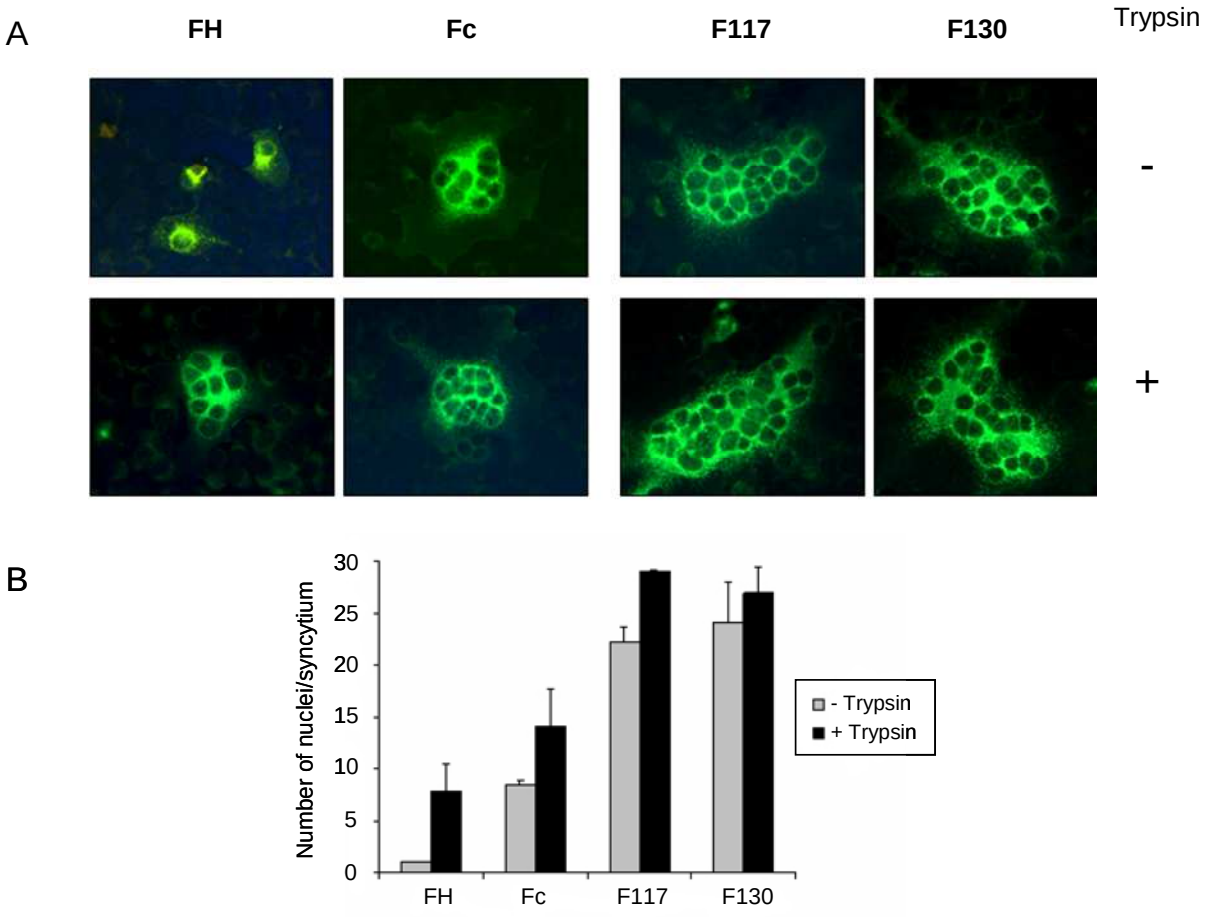


Figure 4.13. Syncytium formation assay. (A) LLC-MK2 cells growing in microchamber wells were infected with SeV-FH, SeV-Fc, SeV-F117 or SeV-F130 viruses at an MOI of 0.5 in the absence or presence of trypsin. Infected cells were processed for syncytia formation 30 h post-infection. (B) The number of nuclei in ten syncytia (chosen at random) in duplicate infections were counted using an AxioCam HRC digital camera (20x magnification). Results are representative of three independent experiments.

4.5.2 Cell content mixing by recombinant Sendai virus mutants

Further quantitation of cell-cell fusion by recombinant viruses was carried out by analysis of cell content mixing using the luciferase reporter gene assay. BSR-T7/5 cells were infected with rSeV (MOI 5), and subsequently mixed (14 h post-infection) with LLC-MK2 cells, which had been previously transfected with a plasmid encoding the luciferase gene (pTM1-Luc). The two populations of cells were left to fuse for 3 h at 37ºC (Fig. 4.14A) or 33ºC (Fig. 4.14B) in the absence or presence of trypsin. Results are expressed as a percentage of wild-type fusion (fusion of SeV-FH in the presence of trypsin). Cell-cell fusion mediated by the single cleavage site SeV-Fc mutant virus was not significantly increased with respect to wt SeV-FH at either 33ºC or 37ºC. In contrast, double cleavage site mutants SeV-F117 and SeV-F130 displayed increased cell-cell fusion, particularly at 33ºC, with SeV-F117 producing the highest level of cell-cell fusion (approximately 3.5 times the level of wt cell fusion in the presence of trypsin at 33ºC). The increased titres seen for double cleavage site mutant viruses, particularly at 33ºC (Table 4.1) may reflect increased cell-cell spread (Figures 4.13 and 4.14). In summary, insertion of double F_RSV cleavage sites in F_SeV leads to an increase in both syncytia size and the extent of cell content mixing.

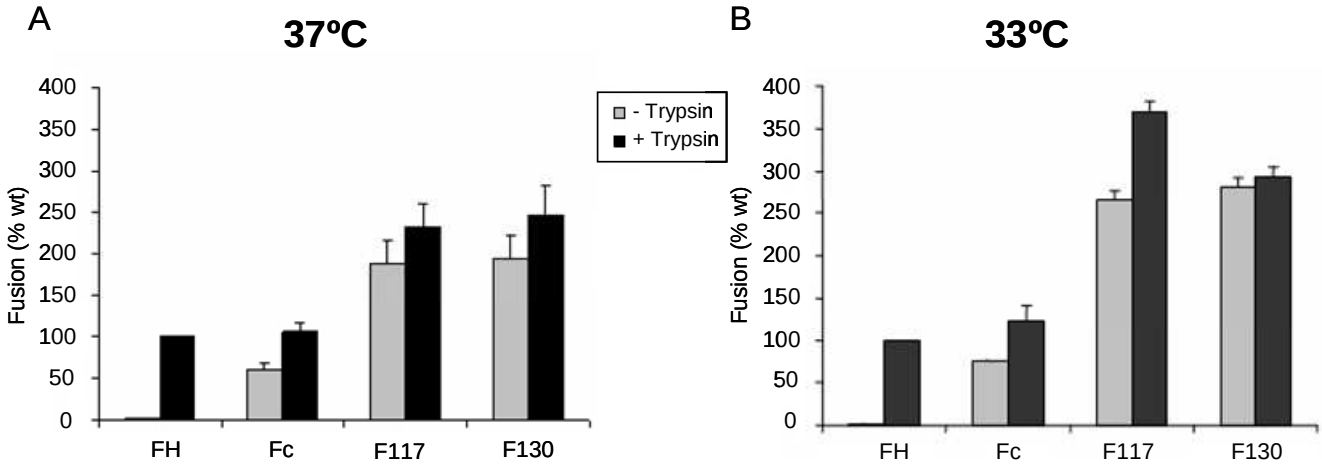


Figure 4.14. Quantitative cell-cell fusion assay. BSR-T7/5 cells were infected with SeV-FH, SeV-Fc, SeV-F117 or SeV-F130 viruses at an MOI of 5 and incubated for 14 h in the absence or presence of trypsin. LLC-MK2 cells, which had previously been transfected with pTM1-Luc, were overlayed onto infected BSR-T7/5 cells 14 h post infection, and incubated in the absence or presence of trypsin for 3 h at 37℃ (A) or 33℃ (B) to allow fusion. Cells were subsequently

lysed and analysed for luciferase activity. Results (relative light units) are expressed as a percentage of wild-type (wt) fusion (SeV-FH + trypsin), with mean values from at least two independent experiments shown.

4.6 Dependence of recombinant Sendai viruses containing fusion protein cleavage site mutations on sialic acid for infection

4.6.1 Effect of neuraminidase treatment on the infectivity of recombinant Sendai virus mutants

Double cleavage site mutant F_SeV proteins were able to fuse transfected cells in the absence of co-expression of the HN attachment protein (Rawling *et al*., 2008). It was therefore of interest to examine the dependency of recombinant Sendai viruses containing mutations at the F protein cleavage site on the interaction between HN and sialic acid for infection. LLC-MK2 cells grown in microchamber culture slides were treated with 120 mU neuraminidase (NA) for 90 min at 37℃ in order to eliminate cell surface sialic acid receptors. Cells were subsequently infected with rSeV at an MOI of 1, and incubated in the absence of trypsin. The extent of infection was revealed by immunofluoresence of fixed cells with MAb GB5 at 20 h post-infection. The level of infection in response to neuraminidase treatment was determined by counting the number of infected cells. As shown in Fig 4.15A, both single cleavage site viruses SeV-FH and SeV-Fc failed to produce significant infection in cells pre-treated with neuraminidase. In contrast, double cleavage site mutant viruses SeV-F117 and SeV-F130 retained the ability to infect neuraminidase-treated cells, producing approximately 40% infection following neuraminidase treatment, compared to approximately 10% infection produced by single cleavage site mutants (Fig. 4.15B).

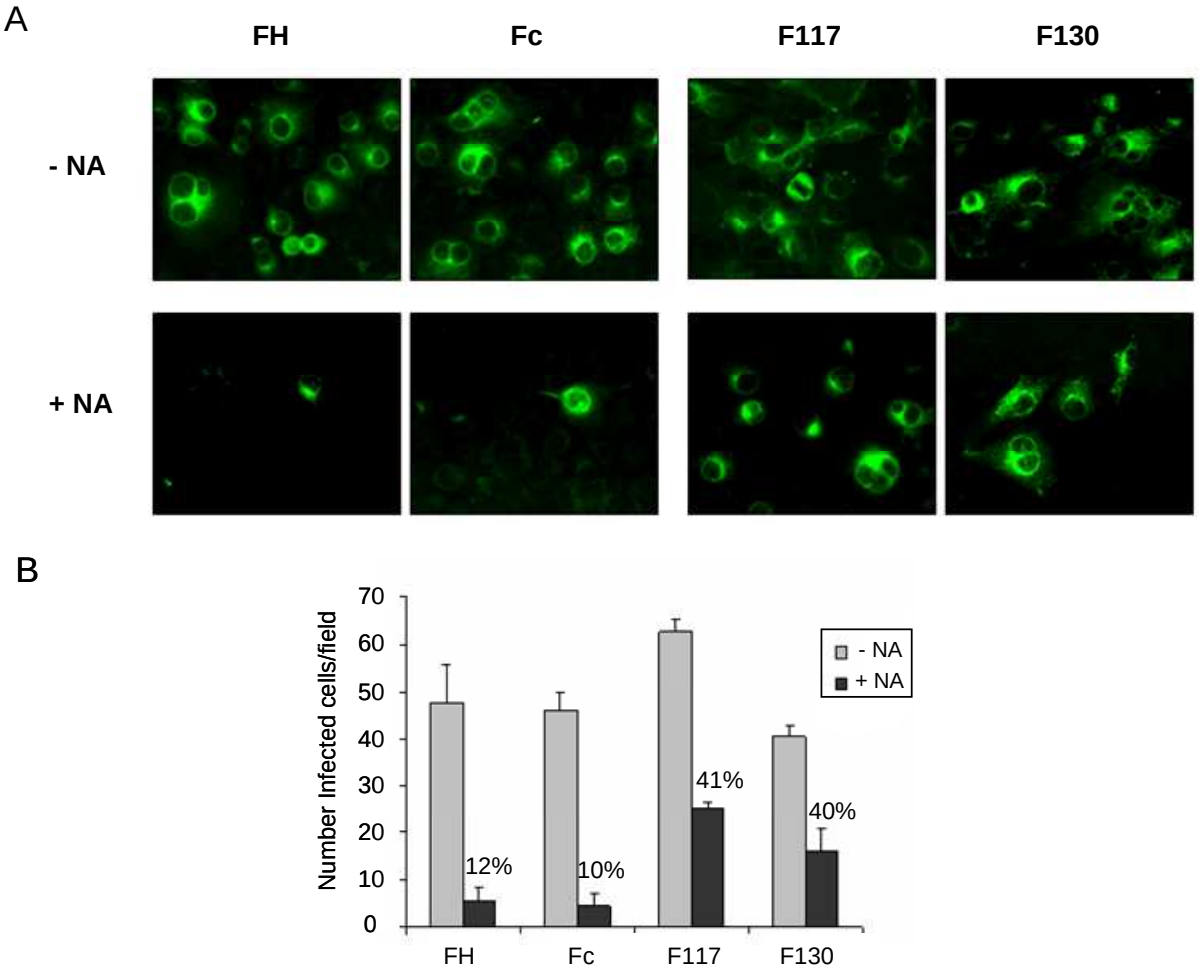


Figure 4.15. Effect of neuraminidase treatment on rSeV infection. (A) LLC-MK2 cells growing in microchamber wells were pre-treated with 120 mU neuraminidase (NA) for 90 min at 37ºC, and subsequently infected with SeV-FH, SeV-Fc, SeV-F117 or SeV-F130 viruses at an MOI of 1. Cells were processed for immunostaining 20 h post-infection. (B) The number of infected cells in five fields (chosen at random) in duplicate infections were counted using an AxioCam HRC digital camera (20x magnification). Also indicated is the percentage infection in NA-treated cells compared to untreated cells. Results are representative of three independent experiments.

4.6.2 Comparison of the ability of recombinant Sendai virus mutants to infect cells devoid of sialic acid receptors

To further examine the ability of double cleavage site mutants to infect cells devoid of sialic acid receptors, we infected a CHO cell line (Lec2), which is defective in sialic acid expression (Stanley *et al*., 1975). Lec2 are a derivative of Pro-5 cells, and display a 90% reduction in the sialylation of glycoproteins due to defective transport of CMP-sialic acid into the trans-Golgi compartment (Deutscher *et al*., 1984; Stanley *et al*., 1980). Lec2 and Pro-5 cells were infected in microchamber culture slides with an MOI of 1 and subjected to immunofluorescent staining at 20 h post infection with the GB5 MAb. Whereas the single cleavage site viruses SeV-FH and SeV-Fc were unable to produce significant infection of Lec-2 cells, both double cleavage site viruses SeV-F117 and SeV-F130 were able to infect Lec2 cells, albeit at a reduced level compared to the level of infection observed in Pro-5 cells (Fig. 4.16A). Syncytia formation was not observed in Lec2 cells, which may reflect the lower level of infection by SeV-F117 and SeV-F130 in this cell type.

In order to quantify the immunofluorescence results, an ELISA was carried out on infected Pro-5 and Lec2 cells at 20 h post infection, using the MAb GB5 directed against SeV F protein (Fig. 4.16B). Single cleavage mutants produced only a low level of infection in Lec2 cells (approximately 5% the level of infection of wt Pro-5 cells), which probably reflects the background level of sialic acid expression in Lec2 cells (Stanley *et al*., 1980). However, double cleavage mutants SeV-F117 and SeV-F130 produced a higher level of infection in Lec2 cells (26% and 28%, respectively). Thus, while infection by double cleavage site mutants is enhanced by conditions that favour an interaction between HN and sialic acid, the presence of two cleavage sites results in a reduced dependency on the HN protein for infection.

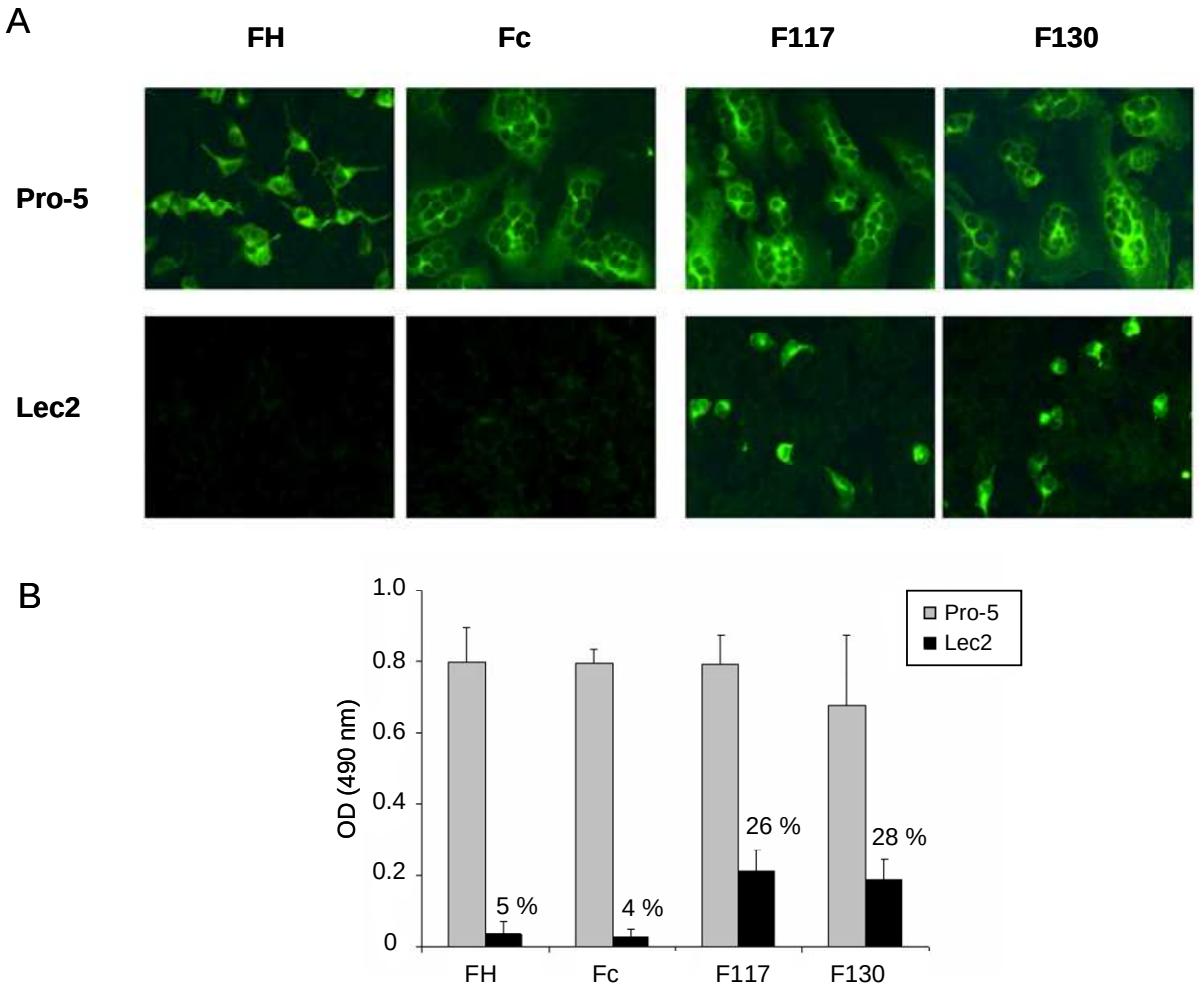


Figure 4.16. Infection of sialic acid-deficient cells. (A) Pro-5 or sialic acid-deficient Lec2 cells growing in microchamber wells were infected with SeV-FH, SeV-Fc, SeV-F117 or SeV-F130 viruses at an MOI of 1. Cells were processed for immunostaining 20 h post-infection. (B) Pro-5 or Lec2 cells growing in 96-well plates were infected with SeV-FH, SeV-Fc, SeV-F117 or SeV-F130 viruses at an MOI of 1 and fixed 20 h post infection. Cells were subsequently stained for expression of SeV F protein with MAb GB5 and the optical density at 490 nm measured in order to quantitate infection. The percentage infection in Lec2 cells relative to Pro-5 cells is indicated. Results represent the mean of three independent experiments performed in duplicate.

5. DISCUSSION

5. DISCUSSION

5.1 Effect of cleavage site mutations on cell-cell fusion mediated by Sendai virus fusion protein

RSV is unique among paramyxoviruses, since the RSV fusion protein (F_RSV) contains two (rather than one) multibasic cleavage sites, and is able to fuse with target cell membranes in the absence of a separate attachment protein. On comparison of cell-cell fusion mediated by RSV and SeV fusion proteins, it was observed that co-expression of the G protein increased cell-cell fusion mediated by F_RSV by approximately two-fold in a quantitative assay (Fig. 4.2B). Therefore, while not a strict requirement, G enhances F_RSV-mediated cell-cell fusion by a mechanism that remains essentially unknown. These results are in good agreement with those of Techaarpornkul *et al*., 2001, who reported a similar level of fusion enhancement by the G protein in recombinant RSV that expressed either F or both F and G proteins. Human metapneumovirus (HMPV) F protein has also been shown to mediate cell-cell fusion (Schowalter *et al*., 2006) and virus-cell fusion (Biacchesi *et al*., 2004) in the absence of the attachment G protein, suggesting that members of the *Pneumovirinae* subfamily have evolved attachment protein-independent mechanisms of F protein activation. In contrast, F_SeV displayed an absolute requirement for both HN co-expression and trypsin for cell-cell fusion, confirming previously published results (Scheid and Choppin 1974).

F_SeV protein contains a single basic cleavage site (R116), whereas F_RSV requires cleavage of two furin-dependent cleavage sites (RARR109 and KKRKRR136) for cell-cell fusion (González-Reyes *et al*., 2001; Zimmer *et al*., 2001a). As shown in Fig. 4.3, a number of chimeras between RSV and SeV fusion proteins were constructed in order to determine whether the differential requirements for fusion exhibited by F_RSV and F_SeV are related to the distinct proteolytic processing pathways of the two proteins (Rawling *et al*., 2008).

Insertion of the complete F_RSV cleavage site II (KKRKRR) in F_SeV to form the Fc mutant augmented cell-cell fusion in an HN-dependent manner, and decreased the dependence on trypsin for fusion (Fig. 4.5A). The presence of six basic residues at the cleavage site of Fc also increased cleavage and cell-cell fusion over the minimal furin recognition sequence present in F_RKR. However, the Fc mutant was still dependent on the co-expression of HN for cell-cell fusion (Fig. 4.5B). Double cleavage site mutants (F110, F117, FΔ and F130) were more susceptible to proteolytic processing by trypsin and formed larger syncytia than single cleavage site mutants (Figs. 4.4 and 4.5). The presence of two cleavage sites may increase F protein proteolytic processing, thereby increasing the number of F protein molecules available for engagement in membrane fusion. Moreover, all double cleavage site F_mutants also fused cells to form syncytia in the absence of the HN protein, thus mimicking the unique properties of F_RSV to fuse cells in the absence of attachment protein co-expression (Fig. 4.5B).

The F117 double cleavage mutant formed the largest syncytia in the absence of HN co-expression. However, in contrast to the syncytium formation assay, the level of HN-independent cell content mixing produced by F117 was lower than that of wild-type F_SeV co-expressed with the HN protein (Fig. 4.6). Similarly, PIV5 F protein mutants (W3A strain) that were found to form syncytia in the absence of HN co-expression did not display cell-cell fusion activity in a quantitative content mixing assay (Horvath *et al*., 1992). It was hypothesised that unstable PIV5 F proteins are able to fuse with neighbouring cells to form syncytia. In contrast, the reporter gene assay involves the interaction between two distinct populations of cells, and may depend to a greater extent on the coordination of attachment and activation of F protein mutants for fusion. Thus, the ability of double cleavage site mutant F_SeV proteins to reproduce the attachment protein-independent phenotype of F_RSV in both syncytium formation and cell content mixing assays suggests that the presence of two multibasic cleavage sites may represent an alternative strategy to regulate the activation of RSV F protein for cell-cell fusion in the absence of a separate attachment protein.

The intervening segment between the two cleavage sites modulated the capacity of the F_SeV protein to fuse membranes, and this modulation appeared greater in the absence of HN. In particular, while the F117 mutant formed large syncytia without HN, the FΔ mutant produced only small syncytia (Fig. 4.5). FΔ is related to F117 by a deletion N-terminal to the second cleavage site, which results in removal of an N-glycosylation motif that has previously been shown to be glycosylated in the Z strain of SeV F protein (Segawa *et al*., 2000). There are three N-glycosylation sites present in pep27, and it has been estimated that at least two of the three sites are glycosylated (Zimmer *et al*., 2001a). Mutation of one site (N126), resulted in increased F_RSV cell-cell fusion, suggesting that differential glycosylation of pep27 may modulate fusion (Zimmer *et al*., 2001b; Zimmer *et al*., 2002). Similarly, it has been demonstrated that an oligosaccharide in the vicinity of the influenza virus HA cleavage site interferes with HA cleavage (Kawaoka and Webster 1989; Deshpande *et al*., 1987) and fusion (Ohuchi *et al*., 1997).

5.2 Effect of fusion protein cleavage site mutations on the fusion and infectivity of recombinant Sendai virus

The results from chimeric F_SeV mutant proteins indicated that the presence of two multibasic cleavage sites may represent a strategy to activate the RSV F protein for cell-cell fusion in the absence of an attachment protein (Rawling *et al*., 2008). However, it has been suggested that cell-cell and virus-cell fusion processes have different structural requirements (Connolly and Lamb 2007). It was therefore of interest to generate recombinant Sendai viruses (rSeV) that expressed mutant F proteins containing one or both F_RSV cleavage sites, separated either by a partial or complete intervening pep27 sequence (Fig. 4.9A; Rawling *et al*., 2011). Rescued rSeV were initially passaged in embryonated chicken eggs, as detailed in Fig. 4.9, in order to increase virus titre. As indicated in the legend of Fig. 4.9, double cleavage site mutants SeV-F117 and SeV-F130 were attained at lower titres following amplification in eggs than single cleavage site mutants SeV-FH and SeV-Fc. However, it is not known if this reflects lower replication in eggs or lower efficiency of rescue of the double cleavage site viruses compared with single cleavage site viruses. In

contrast, while the overall titres obtained in the presence of trypsin after 96 h were similar for all rSeV (Fig. 4.10), double cleavage site rSeV replicated with slightly faster replication kinetics during the first 48 h of replication, in particular at 33℃ (Table 4.1). Thus, it is possible that double cleavage site viruses display greater pathogenicity or stimulate the innate immune response to a greater extent than single cleavage site viruses in embryonated eggs.

Similarly to the results seen in transfected cells, the presence of both F_RSV cleavage sites was found to increase the fusogenicity of rSeV, as measured by both syncytium formation and cell content mixing fusion assays (Figures 4.13 and 4.14). In addition, double cleavage site rSeV were able to infect cells devoid of sialic acid receptors (Figures 4.15 and 4.16), thereby mimicking the unique properties of RSV to fuse and infect cells in the absence of an attachment protein. Cleavage site mutants, in particular the double cleavage site mutant F130, were also found to be less thermostable than wt SeV-FH (Fig. 4.12). It is likely that introduction of basic residues at the F_SeV cleavage site perturbs local protein conformation, thereby reducing thermostability.

Previous studies have reported that mutations at the base of the prefusion head of PIV5 F protein (W3A strain), which result in hyperfusogenicity and HN-independent syncytia formation (Horvath *et al*., 1992), act by destabilising the prefusion conformation of the F protein (Paterson *et al*., 2000; Russell *et al*., 2003; Tsurudome *et al*., 2001). Mutation of equivalent residues in the NDV F protein also resulted in hyperfusogenicity, the promotion of fusion at lower temperatures and cell-cell fusion in the absence of the HN protein, although cell-cell fusion required co-expression of the influenza HA attachment protein (Ayllón *et al*., 2010). Such destabilising mutations may lower the activation energy required to trigger the F protein for fusion, thus reducing dependency on the HN protein for fusion (Connolly *et al*., 2009; Paterson *et al*., 2000). Similarly, the presence of double cleavage sites may reduce the thermostability of the F protein, thereby facilitating F activation for fusion by lowering the energy threshold required to trigger conformational changes involved in fusion. Indeed, our results indicated that the least thermostable mutant (SeV-F130) displayed the greatest ability to infect cells in an HN-independent manner.

However, there was no strict correlation between thermostability (Fig. 4.12) and HN-independence for infection, since the single cleavage site mutant SeV-Fc was less thermostable than the wild-type SeV-FH, but still required sialic acid receptors for infection (Figures 4.15 and 4.16).

Destabilising mutations that resulted in increased cell-cell fusion of the murine leukaemia virus (MLV) env glycoprotein were previously shown to be disadvantageous for virus growth (Lavillette *et al*., 1998). It was hypothesised that unstable env proteins at the cell membrane are continuously replaced by new molecules, which are able to mediate cell-cell fusion. In contrast, such unstable proteins may be prematurely activated in the virus particle before virus-cell contact takes place, thereby inactivating the virus. In contrast, hyperfusogenic double cleavage site mutant rSeV were able to infect cells devoid of sialic acid receptors, thereby reproducing the ability of RSV expressing the F protein in the absence of other cell surface glycoproteins to fuse and infect cells (Karron *et al*., 1997; Techaarpornkul *et al*., 2001; 2002).

It is currently unknown how activation of the mutant F_SeV proteins for fusion could be co-oordinated with attachment of rSeV to target cells in the absence of sialic acid receptors. It is possible that residual binding of HN to NA-treated LLC-MK2 cells (Fig. 4.15) or Lec2 cells (Fig. 4.16) is sufficient for fusion and entry of the less thermostable, double cleavage site mutant rSeV, whereas a higher level of attachment is required for fusion of the more thermostable SeV-FH. Alternatively, the double cleavage site viruses may bind to alternative receptors on the target cells. F_SeV has previously been shown to mediate fusion of virosomes with red blood cells in the absence of the HN protein via an interaction with the asialoglycoprotein receptor (Bagai *et al*., 1993). In addition, virus-like particles that expressed F_SeV protein in the absence of HN protein expression were able to infect cells via the asialoglycoprotein pathway (Leyrer *et al*., 1998), suggesting that the acquired ability of F_SeV to bind to cells permits activation of F_SeV for fusion in the absence of HN co-expression.

The ability of recombinant RSV that lacks the attachment G protein to infect cells suggests that the RSV F protein alone is capable of binding to target cells

in order to mediate fusion. It has been shown that F_RSV binds to cell surface glycosaminoglycans (GAGs), although less efficiently than the attachment G glycoprotein (Feldman *et al*., 2000; Karger *et al*., 2001; Techaarpornkul *et al*., 2002). Indeed, infection by recombinant RSV is enhanced approximately three-fold by the presence of the G protein (Schlender *et al*., 2003; Techaarpornkul *et al*., 2001). Similarly, infection by the double cleavage site rSeV mutants was enhanced by the presence of sialic acid receptors on target cells (Figures 4.15 and 4.16). While the precise region of the F protein involved in the interaction with GAGs has not been mapped, a heparin-binding peptide corresponding to F_RSV cleavage site II and part of the fusion peptide (131_**KKRKRR**FLGFLLGVGSA_147) was able to inhibit both attachment and infection by RSV (Crim *et al*., 2007), suggesting that the cleavage site II region of F_RSV interacts with cell surface GAGs.

The furin-dependent cleavage site of a number of other viruses has also been suggested as a candidate heparan sulfate binding domain (de Haan *et al*., 2008; Klimstra *et al*., 1999; Pasquato *et al*., 2007). Binding of Sindbis virus E2 protein to heparan sulphate correlates with the amount of uncleaved E2 present in the virion (Klimstra *et al*., 1999). Interestingly, the HIV-1 gp160 protein also contains two furin cleavage sites, which are separated by 8 amino acids (KAKR503 and REKR511). While cleavage site REKR503 is inefficiently cleaved, studies using synthetic peptides indicate that binding of the uncleaved REKR503 site to heparin induces conformational changes that expose site KAKR511 for furin cleavage (Pasquato *et al*., 2007). The presence of partially-cleaved F protein from rSeV grown in the absence of trypsin indicates that the uncleaved, multibasic cleavage site II is present on approximately one third of F molecules (Fig. 4.11), and would thus be available to bind to cell surface GAGs. Binding of cleavage site II to GAGs may promote fusion in the absence of a separate attachment protein by facilitating either completion of cleavage of all F molecules within the trimer by furin present at the plasma membrane (Thomas 2002), or the fusion of adjacent, F protein trimers that are already fully-cleaved.

However, only double cleavage site rSeV mutants were able to infect sialic acid-deficient cells, suggesting that furin cleavage site I is also involved in the

binding to GAGs and/or the triggering of fusion. An attractive hypothesis is that the insertion of F_RSV cleavage site I directly mediates attachment to cells. However, a linear peptide corresponding to cleavage site I did not bind to heparin-agarose beads (Crim *et al*., 2007). Alternatively, insertion of cleavage site I may alter the local structure of the F_SeV proteolytic processing region, thereby facilitating the projection of cleavage site II from the surface of the F prefusion structure to bind to target cell GAGs. This hypothesis is supported by previous findings, which suggest that the sequence surrounding the F_SeV cleavage site is important for fusion (Heminway *et al*., 1995). While the conformation adopted by the pep27 region in the prefusion F protein is not known, it is likely to form a glycosylated, surface-exposed loop between the two cleavage sites, which are accessible to exogenous trypsin (Fig. 1.4B). We are currently conducting further experiments to test the binding of cleavage site II to cell surface GAGs (Trento *et al*., unpublished data).

5.3 Fusion and infection of RSV F protein cleavage site mutants

Interactions between RSV and GAGs may be important in natural infection, since heparin has been shown to inhibit the initial tissue culture passage of primary isolates (Teng *et al*., 2001). However, recombinant RSV expressing the F protein as the only surface glycoprotein was able to produce a low level of infection (25%) in cells devoid of GAGs, suggesting that F_RSV also binds to alternative receptor(s), which have yet to be identified (Techaarpornkul *et al*., 2002). Studies using chimeric RSV, in which the human RSV (HRSV) F2 subunit was replaced by the bovine RSV (BRSV) F2, have shown that F2 determines the species-specificity of infection of primary haematopoietic cells. In contrast, RSV G may bind to GAGs in a non-specific manner in order to facilitate access of F2 to specific receptors (Schlender *et al*., 2003).

BRSV F protein is also characterised by double cleavage sites (RAR/KR109 and KKRKRR136), which are separated by an intervening sequence that has the same length as that of HRSV (27 amino acids; pep27), but which lacks sequence identity with human pep27 (Fig. 5.1). Following double cleavage and release of the intervening peptide, it has been suggested that subsequent post-

translational modifications convert BRSV pep27 into a biologically active, tachykinin-like peptide (virokinin), which modulates the host immune response (Valarcher *et al*., 2006; Zimmer *et al*., 2003). However, no such role has been attributed to HRSV pep27, since the HRSV pep27 sequence possesses neither the tachykinin motif (Fig. 5.1) or tachykinin-like activity (Zimmer *et al*., 2003).

Fig. 5.1. Sequence alignment of BRSV and HRSV pep27. The amino acid sequences of bovine (BRSV, strain ATue51908) and human (HRSV, Long strain) respiratory syncytial virus pep27 regions were aligned using the BioEdit Sequence Alignment Editor version 7.0.9.0 (Hall 1999). Amino acids are coloured according to identity and shaded for similarity. The tachykinin-like sequence (FYGLM) found in BRSV is indicated (Zimmer *et al*., 2003).

Mutations of F_RSV cleavage site I in the context of recombinant BRSV (rBRSV) reduced both the replication rate of rBRSV in cell culture and the size of syncytia (Zimmer *et al*., 2002). It was suggested that glycosylated pep27 that remains attached to F2 in the absence of cleavage at site I interferes with conformational changes required for fusion, thus reducing the ability of site I rBRSV mutants to replicate. Accordingly, replication of pep27 deletion mutants (Δ106-130; Zimmer *et al*., 2002 or Δ109-136; König *et al*., 2004) was not significantly affected with respect to wt rBRSV. In contrast, syncytia formation was drastically affected, suggesting that virus-cell and cell-cell fusion processes have different requirements with respect to double cleavage (Zimmer *et al*., 2002). We also found that mutation of F_RSV by deletion of pep27, producing F_Δ108-120 and F_Δ108-132 mutants (Fig. 4.7A), led to an increase in cell-cell fusion activity compared to F_R108/109N (Fig. 4.7C). However, fusion of cells transfected with F_RSV cleavage site mutants was reduced with respect to wt

F_RSV, even in the presence of RSV G co-expression. Since co-expression of the attachment G protein with the F_RSV cleavage site mutants failed to fully restore wild-type levels of fusion, it appears that cleavage site mutation directly interferes with fusion of F_RSV, rather than simply reducing the binding of F_RSV to cells. Given the potential role of double cleavage in attachment protein-independent fusion, it would be of interest to investigate the phenotype of F_RSV cleavage site mutations in the context of a recombinant RSV lacking the attachment G protein.

Zimmer *et al*., (2005) have previously rescued rSeV in which both SeV F and HN proteins were replaced by a modified RSV F protein, consisting of the RSV F protein ectodomain fused to the SeV F protein transmembrane and cytoplasmic domains. Replication of rSeV expressing chimeric F_RSV was independent of trypsin and sialic acid, although rSeV co-expressing HN and chimeric F_RSV produced significantly higher titres. The authors concluded that the HN protein performed an accessory function in the chimeric viruses similar to that of the RSV G protein, which enhances RSV attachment and infection, but which is dispensable for virus replication (Schlender *et al*., 2003; Techaarpornkul *et al*., 2001). Furthermore, Schlender *et al*., (2003) have shown that the RSV F2 region is responsible for species-specific infection of primary cells, which is enhanced in a non-specific manner by the G attachment protein. These results reflect the behaviour of rSeV double cleavage site mutants (SeV-F117 and SeV-F130), which although capable of sialic acid-independent infection, displayed enhanced infection of cells expressing sialic acid receptors for the HN attachment protein (Fig. 4.16). Interestingly, the F2 region employed by Schlender *et al*., (amino acids 1-136) included the double cleavage sites and pep27 region that conferred HN-independent infection properties on rSeV (Rawling *et al*., 2011). Thus, it is possible that binding of the RSV F2 pep27 region to cells determines the species-specificity of RSV infection. While other regions of F_RSV could also contribute to attachment protein-independent fusion, the results presented in this thesis indicate that the double cleavage region of F_RSV may play a crucial role in the unique ability of RSV to fuse and infect cells in the absence of a separate attachment protein.

The correct timing of F activation (triggering) is essential for paramyxovirus entry. Activation of the F protein must only occur in proximity to the target cell membrane to prevent premature inactivation of virus particles. For the *Paramyxovirinae* subfamily it remains to be determined how, following attachment to target cell sialic acid receptors, potential conformational changes in the HN protein co-ordinate subsequent activation of the F protein for fusion. Moreover, it is currently unknown how fusion is triggered for members of the *Pneumovirinae* subfamily, which do no depend on a separate attachment protein for fusion and infection. Interestingly, syncytia formation by the human metapneumovirus F protein expressed alone in transfected cells was dependent on low pH (Schowalter *et al*., 2006), at least for certain strains (Herfst *et al*., 2008). It is therefore possible that members of the *Pneumovirinae* subfamily have evolved distinct mechanisms of F protein activation compared to those of the *Paramyxovirinae* subfamily.

6. CONCLUSIONS

6. CONCLUSIONS

1. Chimeric F_SeV mutants containing one or both F_RSV cleavage sites, separated either by a partial or complete intervening pep27 sequence, were expressed at a similar level to wild-type (wt) F_SeV on the surface of transfected cells.
2. F_SeV mutants with double cleavage sites were more susceptible to proteolytic processing in the presence of trypsin.
3. Double cleavage site F_SeV mutants fused transfected cells to a greater extent than single cleavage site mutants, both in the absence or presence of trypsin.
4. Insertion of both F_RSV cleavage sites in F_SeV reduced dependency on the HN attachment protein for syncytia formation.
5. Recombinant SeV (rSeV) expressing cleavage site mutant F_SeV proteins were rescued to high titres following amplification in embryonated eggs.
6. Double cleavage site mutant rSeV replicated with slightly faster replication kinetics than single cleavage site rSeV, in particular at 33ºC.
7. All rSeV expressing F proteins with furin-dependent cleavage sites were less thermostable than wt rSeV.
8. Double cleavage site rSeV mutants fused infected cells to a greater extent that single cleavage site mutants, in particular at reduced temperatures.
9. rSeV containing two cleavage sites were able to fuse and infect cells lacking sialic acid receptors for the HN attachment protein. Thus, infection by double cleavage site rSeV mutants is less dependent on the attachment protein.
10. Insertion of both F_RSV cleavage sites in F_SeV in the context of rSeV mimics the unique cell-cell fusion and infection properties of RSV.

CONCLUSIONES

1. Los mutantes quiméricos de F_SeV que contienen uno o ambos sitios de corte de F_RSV, separados por una secuencia del pep27 parcial o completa, se expresaron en la superficie de células transfectadas a unos niveles equivalentes a aquellos de F_SeV *wild-type*.
2. Los mutantes de F_SeV con doble sitio de corte fueron más susceptibles al procesamiento proteolítico en presencia de tripsina.
3. Los mutantes de F_SeV con doble sitio de corte exhibieron una mayor capacidad de fusionar células transfectadas, ambos en ausencia o presencia de tripsina.
4. La inserción de los dos sitios de corte de F_RSV en F_SeV reduzcó la dependencia de F de la proteína de unión al receptor (HN) para la formación de sincitios.
5. Virus recombinantes Sendai (rSeV) que expresan distintas proteínas F con mutaciones del sitio de corte fueron rescatados en títulos elevados tras su amplificación en huevos embrionados.
6. Los mutantes de rSeV con doble sitio de procesamiento exhibieron una cinética de replicación ligeramente más rápida que los mutantes con un único sitio de corte, particularmente a 33ºC.
7. Todos los rSeV que expresan las distintas proteínas F con mutaciones del sitio de corte por furina manifestaron menor termoestabilidad que rSeV *wild-type*.
8. Los mutantes rSeV con doble sitio de corte mostraron una mayor capacidad de fusionar células infectadas, particularmente a bajas temperaturas.
9. Los virus rSeV con dos sitios de corte fueron capaces de fusionar e infectar células que carecen de receptores de ácido siálico para la proteína de unión HN. Así, la infección por rSeV que tienen el doble sitio de corte es menos dependiente de la proteína de unión al receptor.
10. La inserción de los dos sitios de corte de F_RSV en F_SeV en el contexto de rSeV se asemejan a las propiedades de fusión célula-célula y de infección de RSV.

7. REFERENCES

8. REFERENCES

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8. APPENDIX

8. APPENDIX

8.1 INTRODUCCIÓN

8.1.1 Clasificación de paramixovirus

La familia *Paramyxoviridae* consiste en una serie de virus envueltos, con un genoma compuesto por una hebra de RNA de polaridad negativa (*Mononegavirales*) e incluye virus humanos de gran prevalencia (sarampión, virus respiratorio sincitial), virus animales de gran importancia económica (virus de la enfermedad de Newcastle, virus de la peste bovina), así como los recientemente identificados virus letales Hendra y Nipah. Los paramixovirus se subdividen en dos subfamilias: *Paramyxovirinae* y *Pneumovirinae* (Collins y Crowe 2007). Los *Paramyxovirinae* y *Pneumovirinae* se distinguen morfológicamente por las diferencias en el tamaño y la forma de sus nucleocápsidas (Bhella y col., 2002), por el número de proteínas que codifican, y por las características de sus proteínas de unión al receptor (Figura 1.1).

El virus Sendai (SeV) y el virus respiratorio sincitial (RSV) son miembros representativos de las subfamilias *Paramyxovirinae* y *Pneumovirinae* respectivamente. RSV se aisló en 1956 de un chimpancé de laboratorio con resfriado común (Blount y col., 1956). Un año más tarde, RSV fue aislado a partir de dos niños con enfermedades respiratorias, y se confirmó mediante estudios serológicos que la infección era común en niños (Chanock y col., 1957). RSV es actualmente reconocido como la causa más importante de enfermedades infantiles graves del tracto respiratorio en el mundo entero (Nair y col., 2010), y puede también provocar enfermedades serias en ancianos y enfermos inmunodeprimidos (Falsey y col., 2005). RSV es considerado un patógeno de interés para el desarrollo de vacunas, sin embargo, la vacunación de recién nacidos con RSV inactivado con formalina (FI-RSV) en los años 60 no produjo protección. Más aún, la vacunación con FI-RSV se asoció con un aumento en la gravedad de la enfermedad en posteriores infecciones naturales con RSV (Openshaw y col., 2001). Por otro lado, la infección natural con RSV no produce protección inmunológica duradera, y las infecciones repetidas a lo

largo de la vida son comunes. Por tanto, los avances en el conocimiento de la biología de RSV son esenciales para el desarrollo de vacunas y agentes antivirales eficaces.

El virus Sendai, también conocido como virus de la parainfluenza murina tipo I, es el responsable de infecciones muy contagiosas del tracto respiratorio en ratones, hamsters y ratas. Este virus se estudia frecuentemente como modelo representativo de la familia *Paramyxoviridae*, con el fin de estudiar las características moleculares y propiedades biológicas de los paramixovirus (Faisca y Desmecht 2007). El interés del estudio de SeV ha aumentado en años recientes debido a su potencial como vector de transferencia génica (Kinoh y Inoue 2008).

8.1.2 Biología molecular de los paramixovirus

8.1.2.1 Genoma y proteínas de los paramixovirus

El genoma de los paramixovirus está formado por una cadena sencilla de RNA no segmentada de una longitud entre 15 y 19 kB. Contiene dos regiones extracistrónicas, una en el extremo 3' de aproximadamente 50 nucleótidos (leader), y otra región en el extremo 5' de 50-161 nucleótidos (trailer), que son esenciales para la transcripción y la replicación. También hay secuencias de control de la transcripción en las regiones anteriores y posteriores a cada gen, que están separados por regiones intergénicas. Las regiones intergénicas de los *Respirovirus* son exactamente 3 nucleótidos, pero su longitud varía en los *Pneumovirus* entre 1-56 nucleótidos. Los genomas de los paramixovirus contienen generalmente 5-10 genes unidos secuencialmente (Fig. 1.1), sin embargo, la capacidad de codificación puede ser amplificada mediante el uso de fases de lectura abierta o *open reading frames* (ORFs) solapantes.

Proteínas de la nucleocápsida N, P, L y M2-1: Los paramixovirus están formados por una nucleocápsida estable central que está rodeada por una envuelta lipídica, que se incorpora a la partícula según el virus emerge de la célula huésped (Fig. 1.2). La nucleoproteína (N) se une al RNA genómico

(polaridad -) y antigenómico (polaridad +) para formar el molde de la nucleocápsida helicoidal, que es la forma biológicamente activa del RNA viral (vRNA). La estructura cristalina de un complejo de la proteína N de RSV reveló que la proteína es capaz de formar anillos decaméricos que se unen al RNA a través de una hendidura o *groove* formada por residuos básicos (Tawar y col., 2009). Por otro lado, N se une también a la fosfoproteína (P) y a la polimerasa (L) para formar el complejo ribonucleoproteína (RNP), que representa la unidad mínima de replicación (Grosfeld y col., 1995; Yu y col., 1995). La proteína L es una RNA polimerasa viral dependiente de RNA (vRNAP), que se encarga de la transcripción del mRNA viral funcional, incluyendo el *capping* y la metilación del extremo 5' y la poliadenilación del extremo 3' (Ogino y col., 2005). La proteína L se encuentra en muy pequeña concentración en las células infectadas. Por ejemplo, cada nucleocápsida del virus Sendai está compuesta de aproximadamente 2600 moléculas de N, 300 de P y 50 de L (Lamb y col., 1976). Mientras que la proteína L posee toda la actividad catalítica del vRNAP, L se une al molde de la nucleocápsida por medio de la proteína P (Horikami y col., 1992). La proteína P está fosforilada en residuos de Ser/Thr, y es un cofactor esencial para el funcionamiento de las proteínas L y N. Finalmente, la proteína M2-1 (específica de los neumovirus), se incorpora también a la nucleocápsida. Se transcribe desde el primer ORF del gen M2 y actúa como un factor esencial para facilitar la transcripción (Collins y col., 1995).

Proteína matriz M: Como muestra la Figura 1.2, la proteína matriz (M) recubre la cara interna de la envuelta. La proteína M es la más abundante en el virión y se cree que juega un papel central en la mofogénesis viral, mediante las interacciones con la cola citoplasmática de las glicoproteínas de la envuelta, la bicapa lipídica y la nucleocápsida. La capacidad de la proteína M de autoasociarse, combinada con la interacción que se produce con la nucleocápsida, puede ser responsable de la escisión por gemación de los viriones (Ghildyal y col., 2006).

Glicoproteínas de la envuelta HN/H/G, F y SH: Los paramixovirus contienen dos glicoproteínas integrales de membrana dentro de la envuelta lipídica, codificadas por el genoma viral: la proteína de fusión (F) y la de unión al

receptor (glicoproteína [G], hemaglutinina [H], o hemaglutinina-neuraminidasa [HN]), que forman espículas de 8-12 nm de la superficie del virion (Fig. 1.2). Todos los paramixovirus poseen una proteína de fusión, que facilita la fusión de las membranas celular y viral durante la entrada del virus. Sin embargo, las características de la proteína de unión, que es la responsable de la unión del virus a la superficie de la célula diana, varían considerablemente entre las dos subfamilias de los *Paramyxoviridae*. Las glicoproteínas de fusión y unión al receptor de los paramixovirus representan importantes dianas antigénicas, ya que inducen la producción de anticuerpos neutralizantes (Walsh y col., 1987). Dado que el interés de esta tesis se centra en la entrada de los paramixovirus en la célula, las proteínas de fusión y de unión serán descritas en detalle en la sección 1.3.

El virus de las paperas, RSV y PIV5 poseen una tercera proteína de membrana de función desconocida: la proteína hidrofóbica pequeña (SH). La proteína SH no es imprescindible para la replicación del virus RSV en cultivos celulares, aunque su delección de un RSV recombinante resultó en la atenuación del crecimiento viral (Bukreyev y col., 1997). Las proteínas SH de los paramyxovirus inhiben la señalación TNF-α y la apoptosis de células infectadas, lo que podría favorecer la infección (Fuentes y col., 2007; Wilson y col., 2006). Un análisis por microscopía electrónica de la proteína SH de RSV reveló unas estructuras en forma de anillo pentamérico o hexamérico con un poro central, que permitiría el paso de iones y moléculas pequeñas (Carter y col., 2010).

Proteínas accesorias: El gen P/V/C de los paramixovirus dirige la expresión de una serie de proteínas accesorias (V, W, C', C, Y1 y Y2 para SeV) mediante la edición del RNA y el uso de codones de traducción alternativos. Aunque las proteínas accesorias no son esenciales para la infección, ejercen diversas funciones en el ciclo de crecimiento viral, incluyendo el control de la síntesis de RNA y la inhibición de los componentes celulares de la respuesta de interferon de tipo I (Goodbourn y Randall 2009). Los neumovirus son únicos dentro de la familia *Paramixovirinae*, ya que no producen proteínas alternativas del gen de la P. Sin embargo, RSV tiene dos genes adicionales que codifican dos

proteínas no estructurales (NS1 y NS2), que son capaces de inhibir la indución de interferon de tipo I en respuesta a las infecciones virales (Spann y col., 2004). Estas proteínas promueven el crecimiento de RSV, pero no son esenciales para su replicación *in vivo* (Teng y col., 1999; 2000).

8.1.2.2 Ciclo Replicativo

El ciclo replicativo completo de los *Paramyxoviridae* tiene lugar en el citoplasma de la célula diana (Fig.1.3). La entrada viral requiere primero la unión de los viriones a la superficie celular. Para los miembros de los géneros *Rubulavirus* y *Respirovirus*, los receptores celulares son moléculas que contienen ácido siálico. Para la proteína de unión al receptor (HN) del virus Sendai, los receptores son gangliósidos que contienen ácido siálico (Markwell y col., 1980, 1984). En la mayoría de los neumovirus el receptor es desconocido, sin embargo, RSV interacciona con los glicosaminoglicanos de la superficie celular, en particular con heparán sulfato (Escribano-Romero y col., 2004; Feldman y col., 1999, 2000; Hallak y col., 2000; Karger y col., 2001). Tras la unión, la proteína de fusión (F) de los paramixovirus induce la fusión de la envuelta viral con la membrana plasmática celular a pH neutro.

La fusión de las membranas viral y celular permite la entrada de la nucleocápsida en el citoplasma, donde tiene lugar la transcripción del genoma viral por la vRNAP, dando lugar a un conjunto de mRNAs que son traducidos por los ribosomas celulares. La vRNAP transcribe el molde genómico de polaridad negativa de manera secuencial a partir de un único promotor en el extremo 3’ del RNA para generar mRNAs poliadenilados y con estructura de *cap*. La transcripción se inicia en la señal de comienzo del primer gen (GS o gene start) en el extremo 3’ y procede a lo largo del genoma molde hasta que se alcanza la secuencia de final del gen (GE o gene end). El vRNAP escanea posteriormente las regiones intragénicas sin disociarse del genoma modelo, reiniciando la transcripción en el siguiente GS mediante un mecanismo *start-stop* de transcripción. Esto da como resultado un gradiente polar 3’-5’ de mRNA, debido a que la probabilidad de que la polimerasa inicie la transcripción en los subsiguientes GS va disminuyendo según alcanza nuevas regiones

intragénicas. Tras la traducción de los tránscritos primarios y la acumulación de proteínas virales, el genoma viral se replica para producir un antigenoma completo de polaridad positiva (cRNA). El antigenoma representa una molécula intermedia en la replicación genómica, acomplejándose con la proteína N y actuando como molde para la posterior síntesis de genomas progenie. La replicación eficiente del RNA de SeV depende de la llamada "regla del seis", que consiste en que la longitud del genoma viral debe ser múltiplo de seis para replicarse de forma eficiente (Calain y Roux 1993). Por el contrario, la replicación del genoma de RSV no sigue la regla del seis (Samal y Collins 1996). De forma similar a otros virus con envuelta, los paramixovirus emergen de la membrana plasmática de la célula infectada en los sitios donde los componentes virales se han ensamblado. Finalmente, las gemas se escinden, resultando en la liberación de las nuevas partículas virales.

8.1.3 Glicoproteínas de la envuelta de los paramixovirus

8.1.3.1 La proteína de fusión

Las proteínas de fusión de los paramixovirus son las responsables de la entrada del virus, promoviendo la fusión entre la envuelta viral y la membrana plasmática celular a un pH neutro. Además, las proteínas F que se expresan en la membrana plasmática de las células infectadas pueden dar lugar a la fusión entre células vecinas para formar sincitios multinucleados. A pesar de la escasa identidad de secuencia entre ellas, las proteínas F de los paramixovirus comparten muchos elementos estructurales, incluyendo la localización de residuos cisteínas, glicinas y prolinas, lo que sugiere una estructura similar para todas. En la Figura 1.4A se muestra como la proteína F tiene tres regiones hidrofóbicas principales: un péptido señal (SP) N-terminal que dirige la proteína al retículo endoplásmico durante su biosíntesis, una región transmembranal (TM) en la región C-terminal y un péptido de fusión (FP) situado en el extremo N-terminal de la cadena F1. El péptido de fusión se inserta en la membrana de la célula diana durante el proceso de fusión de membranas (Russell y col., 2001). La proteína F de los paramixovirus contiene dos regiones con secuencias heptádicas (4-3) repetidas, HRA y HRB, situadas adyacentes al

péptido de fusión y al dominio transmembranal respectivamente. Estas regiones tienen un papel crucial en el mecanismo de fusión de membranas (ver sección 1.3.3).

La proteína F de los paramixovirus es un homotrímero (Russell y col., 1994) que pertenece a la clase I de la familia de proteínas virales de fusión, que comprende entre otras la proteína hemaglutinina (HA) del virus de la influenza, la proteína de la envuelta gp160 del virus HIV, y la proteína G del virus Ébola. La fusión de membranas tiene lugar por medio del replegamiento de una forma prefusión metaestable de la proteína F (Figura 1.4B) a una forma posfusión más estable (Figura 1.4C), pasando por una serie de cambios conformacionales (revisión en Lamb y Jardetzky 2007). Dichos cambios conformacionales resultan en la disposición de las regiones heptádicas de la proteína F (HRA y HRB) en un haz de 6 hélices o *6-helix bundle* (6HB), que es característico de la estructura de postfusión (Figura 1.4C). La estructura 6HB forma una superhélice (*coiled-coil*), que contiene un núcleo interno de tres hélices HRA rodeadas por tres hélices HRB antiparalelas (Baker y col., 1999; Zhao y col., 2000). Esta disposición obliga a situarse al péptido de fusión y al dominio transmembranal en el mismo extremo de la proteína, y se cree que esto está relacionado directamente con la fusión de las membranas viral y celular, dado que la energía libre que se desprende en la formación del 6HB puede utilizarse para la fusión de las membranas (Melikyan y col., 2000; Russell y col., 2001).

La proteína F se sintetiza como un precursor inactivo (F0), que requiere un procesamiento proteolítico que da lugar a dos cadenas polipeptídicas (F1 y F2), unidas por puentes disulfuro (revisión en Lamb y Jardetzky 2007). El corte proteolítico es un requerimiento imprescindible para la activación de la proteína F. El procesamiento proteolítico tiene lugar en un sitio mono- o multibásico que se encuentra adyacente al péptido de fusión, y es reconocido respectivamente por proteasas del tipo tripsina o tipo furina. Las características del sitio de corte son claves para la patogenicidad viral, ya que se ha demostrado que la adquisición de un sitio de corte multibásico está relacionado con mayor patogenicidad e infección sistémica (Klenk y Garten 1994; Nagai y Klenk 1977).

La Tabla 1.1 muestra una comparación de secuencias de los sitios de corte de proteínas F representativas de los paramixovirus. La proteasa intracelular furina, de la familia de las subtilisinas, es la responsable del procesamiento de proteínas con sitios multibásicos, incluyendo la F de RSV (F_RSV). Por el contrario, la proteína F del virus Sendai (F_SeV) contiene un único aminoácido básico en el sito de corte (R116), y requiere la adición de tripsina a los cultivos celulares para que puedan tener lugar sucesivas rondas de replicación (Scheid y Choppin 1974; Kido y col., 1999). Se cree que la proteasa triptasa Clara, que se secreta de las células Clara del epitelio bronquial, es la responsable de la activación de las proteínas con sitios de corte monobásicos en el tracto respiratorio del huésped murino (Kido y col., 1997; 1999).

F_RSV es único entre los *Paramyxoviridae*, ya que F_RSV contiene dos sitios de corte por furina (sito I, RARR109 y sitio II, KKRKRR136), que están separados por una región de 27 aminoácidos (pep27). Los dos sitios de corte y la longitud de pep27 (pero no su secuencia) están altamente conservados en todas las cepas de RSV bovinas y humanas. El procesamiento proteolítico en los dos sitios es necesario para que F_RSV forme sincitios en células transfectadas (González-Reyes y col., 2001; Zimmer y col 2001a). Mientras que la F_RSV no necesita la co-expresión de la proteína de unión para la fusión de membranas (ver sección 8.1.3.4), se requiere el doble procesamiento y la liberación de pep27, que se secreta al medio (Ruiz-Argűello y col., 2002). La existencia de dos sitios proteolíticos en F-RSV es única entre los paramixovirus, aunque se han observado dos sitios de corte en la proteína “spike” (S) del coronavirus SARS (Belouzard y col., 2009).

No se conoce en profundidad la función del doble procesamiento y de la liberación de pep27, pero se sabe que el procesamiento está asociado con un cambio de forma de la proteína F_RSV (Melero 2007; Ruiz-Argűello y col., 2002). En el caso de la proteína F del RSV bovino, se ha sugerido un papel de modulación inmunológica para pep27 (Zimmer y col., 2003), sin embargo dicha función no se ha podido atribuir al péptido de RSV humano, que además no tiene similitud de secuencia con su homólogo bovino.

Las estructuras cristalinas de las formas pre- y postfusión de tres proteínas F de los paramixovirus se han resuelto recientemente, mostrando diferencias conformacionales sustanciales entre ambas estructuras (Swanson y col., 2010; Yin y col., 2005; 2006). Se han modelado las estructuras tridimensionales de las formas pre- y postfusión de la proteína F de RSV a partir de las estructuras publicadas de la forma prefusión del HPIV5 (Fig. 1.4B) y la forma postfusión del HPIV3 (1.4.C). La forma global de la estructura prefusión es la de una cabeza globular unida a un tallo cilíndrico formado por una triple hélice superenrollada que contiene las regiones HRB. La cabeza globular está formada por las regiones HRA y por el péptido de fusión, que está escondido entre las subunidades de la cabeza globular. La localización del péptido de fusión se asemeja a la de la estructura prefusión del virus de la influenza HA (Skehel y Wiley, 2000). Aunque su conformación no se conoce, se cree que la región glicosilada que se encuentra entre los dos sitios de corte (pep27), forma un *loop* accesible al solvente. Como se observa en la Fig. 1.4.C, la forma postfusión puede relacionarse con la forma prefusión mediante un giro del dominio que forma el tallo, respecto de la cabeza. Durante el proceso de fusión (ver sección 8.1.3.3), la región HRA se pliega en una conformación de alfa-hélice sencilla extendida (*coiled coil*) que interacciona con los dominios HRB para dar lugar a la estructura 6HB postfusión.

8.1.3.2 Proteínas de unión al receptor de paramixovirus

Las proteínas de unión al receptor de los paramixovirus (HN, H, o G, según el virus) pueden variar ampliamente en cuanto a su estructura y en cuanto al receptor al cual se unen. El virus Sendai, junto con otros miembros de los géneros *Respirovirus* y *Rubulavirus*, posee una proteína de unión con actividad hemaglutinina-neuraminidasa (HN). Se cree que la unión del virus a la célula diana tiene lugar mediante la unión de HN a receptores de la membrana celular que tienen ácido siálico, mientras que la actividad neuraminidasa de HN podría prevenir la agregación entre partículas virales emergentes (revisión en Villar y Barroso 2006). La proteína HN también promueve la fusión, y se requiere la co-expresión de proteínas F y HN homotípicas para la fusión de la mayoría de los paramixovirus (Horvath y col., 1992). Se ha propuesto que la union de la

protein HN al receptor induce cambios conformacionales que activan o disponen la proteína F para la fusión (Rusell y col. 2001).

La proteína HN (Figura 1.5) es una proteína integral de membrana de clase II, que se compone de una región de anclaje a membrana (TM) que actúa como un péptido de señal para dirigir la proteína al retículo endoplásmico durante su biosíntesis, un dominio del tallo próximo a la membrana y un dominio con forma de cabeza globular C-terminal que contiene las actividades de unión al receptor y la actividad de neuraminidasa (Thompson y Portner 1987). La estructura de la cabeza globular (Figura 1.5B) tiene la conformación de propulsor de seis láminas o *six-blade propeller,* típica de otras estructuras de neuraminidasa (Crennell y col., 2000; Lawrence y col., 2004). Inicialmente se pensó que las dos actividades se localizaban en el mismo sitio, sin embargo distintos estudios de las proteínas HN de NDV y HPIV3 sugieren la presencia de un segundo sitio de unión a ácido siálico en la superficie de interacción entre dímeros (Porotto y col. 2006, 2007; Zaitsev y col. 2004). Para NDV, se ha postulado que la unión de ácido siálico al primer sitio activa el segundo, prolongando la unión del virus a la célula diana (Porotto y col. 2006).

La proteína HN forma homotetrámeros a través de la región del tallo (Yuan y col., 2005), que también puede interaccionar con la proteína F para promover la fusión (ver sección 8.1.3.4). Estudios que utilizan proteínas HN mutantes han demostrado que esta function de la proteína HN como activadora de la proteína F reside en el dominio del tallo (Porotto y col., 2003). Se cree que el equilibro entre las propiedades de la HN como proteína de unión al receptor y regulador de la activación de F regula la entrada del virus.

La estructura de la proteína de unión al receptor de los neumovirus (G), (Figura 1.5) difiere ampliamente de la de las proteínas de unión HN de los paramixovirus. La proteína G carece de actividades hemaglutinina y neuraminidasa y no se une al ácido siálico. La proteína G de RSV es una proteína integral de membrana de tipo II con un único dominio hidrofóbico que funciona como péptido señal y como dominio transmembranal. La proteína G tiene dos dominios altamente glicosilados parecidos a mucina, y migra en SDS-

PAGE con una movilidad mucho menor que la predicha por su peso molecular, debido a su extensa O-glicosilación. A diferencia de lo que sucede con la proteína HN de los paramixovirus, la proteína G de los neumovirus no es necesario para la fusión célula-célula o virus-célula (Biacchesi y col., 2004; Karron y col., 1997; Schmidt y col., 2002; Schowalter y col., 2006; Techaarpornkul y col., 2001, 2002).

Como se muestra en el modelo de la figura 1.5C, se ha sugerido que los dos dominios del tipo mucina formen regiones extendidas no plegadas, separadas por una región central de 13 aminoácidos que se encuentra conservada y que carece de sitios de glicosilación. Esta region contiene cuatro cisteínas conservadas que forman dos puentes disulfuro, resultando en un bucle de cisteína o *cystine noose*. Aunque la region central no es necesaria para la replicación eficiente de RSV, puede tener un papel inmunoregulador (Polack y col. 2005). Un segmento corto adyacente al bucle de cisteínas se ha identificado como un dominio de union a heparina (HBD), que facilita la unión de la protein G a los glicosaminoglicanos (GAGs) de la superficie celular.

La proteína G se produce en dos formas (i) una proteína transmembranal de tipo II (Gm), que se incorpora a la envuelta del virión a través de una region hidrofóbica cercana al extremo N-terminal, y (ii) una proteína soluble (Gs), que se produce por una iniciación alternativa de la traducción, originando una proteína sin región de anclaje a la membrana, por lo que es secretada por las células infectadas. Mientras que Gm forma homo-oligómeros (probablemente tetrámeros), Gs es monomérica, lo que sugiere que la región transmembranal es necesaria para la oligomerización (Escribano-Romero y col. 2004). Aunque Gm es responsable de la union a los GAGs de la célula diana durante la entrada del virus, se ha propuesto para Gs una function inmunomoduladora o como cebo de anticuerpos.

8.1.3.3 Modelo de la fusión de membranas de los paramixovirus

Por estudios realizados con péptidos de las regiones heptádicas, se ha mostrado que durante los cambios conformacionales que tienen lugar durante el proceso de fusión, las regiones HRA y HRB están expuestas antes de que se alcance la estructura termoestable final de 6HB (Russell y col., 2001). La obtención posteriormente de la estructura cristalina de las formas pre- y posfusión de la proteína F dio lugar a una hipótesis para el mecanismo de fusión de membrana que se resume en la figura 1.6 (Yin y col., 2006). En la primera etapa, las hélices HRB se separan desde la base de la cabeza en su conformación prefusion (1.6b). Este intermedio de tallo abierto o *open stalk* se pliega inmediatamente en un intermedio de pre-horquilla o *prehairpin*, en el cual se forma la estructura superhelicoidal (*coiled-coil*) de las regiones HRA debido a la traslocación del péptido de fusión en dirección a la membrana de la célula diana (1.6c). La reestructuración final conduce a la fusión (1.6d), para lo cual la región transmembranal y el péptido de fusión quedan insertados en la misma membrana (1.6e).

8.1.3.4 Función de la proteína de union al receptor en la fusión de los paramixovirus

Se ha propuesto un mecanismo por el cual la presencia de HN rebaja la energía de activación requerida para la fusión de membranas por la proteína F (Paterson y col., 2000). La interacción entre F y HN se ha demostrado en la superficie de células transfectadas (Deng y col., 1999) o infectadas (Stone-Huslander y Morrison 1997). Algunos estudios realizados con proteínas HN quiméricas sugieren que las regiones del tallo y de la cabeza de HN participan en las interacciones con la proteína F (Deng y col., 1995; Tanabayashi y Compans 1996; Tsurudome y col., 1995). Se han propuesto modelos alternativos para explicar la manera en la que la proteína HN dispara la activación de la proteína F (Connolly y col, 2009). En el primero (modelo “pinza”), HN interacciona con la proteína F para estabilizarla en un estado metaestable antes de unirse a la célula diana. Los cambios conformacionales que sufre la protein HN a causa de su union al ácido siálico de la célula

producen la liberación de la proteína F. Según un modelo alternativo (modelo "provocador") son los cambios conformacionales sufridos por HN tras su unión al receptor los que desencadenan la fusión por la desestabilización de la proteína F. De acuerdo con el segundo modelo, la interacción entre HN y F podría ocurrir antes o después de la unión al receptor. Sin embargo, la naturaleza de los cambios conformacionales que sufre HN y su efecto sobre la proteína F aún no se han determinado.

Mientras que la proteína F de SeV, junto con la mayoría de los miembros de la subfamilia *Paramyxoviridae* requiere co-expresión de la proteína de unión HN para fusionar células en cultivo, F_RSV es capaz de fusionar membranas en ausencia de la proteína de unión G. Mutantes espontáneos o recombinantes de RSV manipulados genéticamente que expresan F como única glicoproteína de superficie son capaces de infectar células y formar sincitios (Karron y col., 1997; Schmidt y col., 2002; Techaarpornkul y col., 2001, 2002). Por otro lado, la expresión de la proteína F_RSV como única proteína viral en células transfectadas es suficiente para inducir la formación de sincitios (González-Reyes *et al*., 2001; Zimmer *et al*., 2001a). F_RSV se une a glicosaminoglicanos (GAGs) de tipo del heparán sulfato, (Feldman y col., 2000; Techaarpornkul y col., 2002), lo que puede representar una interacción inicial no específica que permitiría la unión posterior de F_RSV a un receptor específico que active la proteína F para la fusión en ausencia de la proteína G (Feldman y col., 2000). La proteína F del metaneumovirus humano (HMPV) también induce la formación de sincitios en células transfectadas en ausencia de proteína G, siendo este proceso dependiente de pH ácido en algunos cepas (Herfst y col., 2009; Schowalter y col., 2006, 2009). En definitiva, no se conoce el papel exacto de las proteínas de unión al receptor de los miembros de la subfamilia *Pneumovirinae* en el proceso de fusión, pero todo parece indicar que las proteínas F de los neumovirus poseen mecanismos alternativos para la unión al receptor y la fusión de membranas.

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Insertion of the Two Cleavage Sites of the Respiratory Syncytial Virus Fusion Protein in Sendai Virus Fusion Protein Leads to Enhanced Cell-Cell Fusion and a Decreased Dependency on the HN Attachment Protein for Activity▽

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Cell entry by paramyxoviruses requires fusion of the viral envelope with the target cell membrane. Fusion is mediated by the viral fusion (F) glycoprotein and usually requires the aid of the attachment glycoprotein (G, H or HN, depending on the virus). Human respiratory syncytial virus F protein (F_{RSV}) is able to mediate membrane fusion in the absence of the attachment G protein and is unique in possessing two multibasic furin cleavage sites, separated by a region of 27 amino acids (pep27). Cleavage at both sites is required for cell-cell fusion. We have investigated the significance of the two cleavage sites and pep27 in the context of Sendai virus F protein (F_{SeV}), which possesses a single monobasic cleavage site and requires both coexpression of the HN attachment protein and trypsin in order to fuse cells. Inclusion of both F_{RSV} cleavage sites in F_{SeV} resulted in a dramatic increase in cell-cell fusion activity in the presence of HN. Furthermore, chimeric F_{SeV} mutants containing both F_{RSV} cleavage sites demonstrated cell-cell fusion in the absence of HN. The presence of two multibasic cleavage sites may therefore represent a strategy to regulate activation of a paramyxovirus F protein for cell-cell fusion in the absence of an attachment protein.

Human respiratory syncytial virus (RSV) and Sendai virus (SeV) are members of the *Pneumovirinae* and *Paramyxovirinae* subfamilies, respectively, within the *Paramyxoviridae* family of negative-stranded RNA viruses (*Mononegavirales*) (5). Cell entry by paramyxoviruses requires fusion of the viral envelope with the target cell membrane at the cell surface. Paramyxovirus-infected cells are also capable of fusing with adjacent cells to form syncytia (multinuclear, giant cells). Fusion in both cases is mediated by viral envelope glycoproteins, namely, the fusion (F) protein, a trimeric type I integral membrane glycoprotein.

The F polypeptide is synthesized as an inactive precursor (F0), which requires proteolytic cleavage to yield fusion-competent, disulfide-linked F2-F1 chains (for a review see reference 21). Cleavage takes place at either a mono- or multibasic (RXK/RR) cleavage site, recognized by trypsin- or furin-like proteases, respectively, immediately upstream of a hydrophobic fusion peptide that is inserted into the cell membrane during fusion (30). Cleavage is an absolute requirement for fusion, and indeed the nature of the cleavage motif has been shown to play a role in the pathogenicity of some paramyxoviruses (17).

Whereas for most paramyxoviruses, fusion mediated by the F glycoprotein requires participation of the homotypic attachment protein (G, H, or HN, depending on the virus), the F protein of human respiratory syncytial virus is able to fuse membranes in the absence of the attachment G protein. Spontaneous mutants (15) or genetically engineered recombinant RSVs expressing F as the only surface glycoprotein (38, 39) are able to infect cells and form syncytia. Furthermore, expression of the RSV F protein as the only viral protein in transfected cells is sufficient to induce syncytium formation (12, 45).

RSV F protein (F_{RSV}) is unique among the *Paramyxoviridae*, since F_{RSV} possesses two furin cleavage sites (site I, $RARR_{109}$, and site II, $KKRKRR_{136}$), which are separated by a region of 27 amino acids (pep27), as shown in Fig. 1A, below. The two cleavage sites and the length, but not the sequence, of pep27 are highly conserved in all bovine and human RSV strains. Proteolytic processing at both cleavage sites is required by F_{RSV} to form syncytia in transfected cells (12, 45). Thus, while membrane fusion by F_{RSV} does not depend on coexpression of an attachment protein, there is a requirement for double cleavage of the F protein and removal of the intervening pep27, which is secreted into the medium (28). The function of the double cleavage and subsequent pep27 removal are currently unknown, although completion of cleavage is associated with a change in shape of the RSV F protein (25, 28). An immunomodulatory role has been suggested for bovine RSV pep27 (48); however, no such role has been attributed to the human RSV intervening peptide, which does not share sequence similarity with its bovine counterpart.

Despite low sequence identity, paramyxovirus F proteins share structural elements, including the location of cysteine residues, hydrophobic domains, and heptad repeats (HR) A and B, as shown in Fig. 1A, below. Crystallographic data indicate that refolding of the F protein during fusion results in the

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Recombinant Sendai Viruses Expressing Fusion Proteins with Two Furin Cleavage Sites Mimic the Syncytial and Receptor-Independent Infection Properties of Respiratory Syncytial Virus▽

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Cell entry by paramyxoviruses requires fusion between viral and cellular membranes. Paramyxovirus infection also gives rise to the formation of multinuclear, fused cells (syncytia). Both types of fusion are mediated by the viral fusion (F) protein, which requires proteolytic processing at a basic cleavage site in order to be active for fusion. In common with most paramyxoviruses, fusion mediated by Sendai virus F protein (F_{SeV}) requires coexpression of the homologous attachment (hemagglutinin-neuraminidase [HN]) protein, which binds to cell surface sialic acid receptors. In contrast, respiratory syncytial virus fusion protein (F_{RSV}) is capable of fusing membranes in the absence of the viral attachment (G) protein. Moreover, F_{RSV} is unique among paramyxovirus fusion proteins since F_{RSV} possesses two multibasic cleavage sites, which are separated by an intervening region of 27 amino acids. We have previously shown that insertion of both F_{RSV} cleavage sites in F_{SeV} decreases dependency on the HN attachment protein for syncytium formation in transfected cells. We now describe recombinant Sendai viruses (rSeV) that express mutant F proteins containing one or both F_{RSV} cleavage sites. All cleavage-site mutant viruses displayed reduced thermostability, with double-cleavage-site mutants exhibiting a hyperfusogenic phenotype in infected cells. Furthermore, insertion of both F_{RSV} cleavage sites in F_{SeV} reduced dependency on the interaction of HN with sialic acid for infection, thus mimicking the unique ability of RSV to fuse and infect cells in the absence of a separate attachment protein.

Human respiratory syncytial virus (RSV) and Sendai virus (SeV) are enveloped, negative-strand RNA viruses that belong to the *Pneumovirinae* and *Paramyxovirinae* subfamilies, respectively, of the *Paramyxoviridae* (6). Cell entry by paramyxoviruses requires virus binding to target cell receptors, followed by fusion of the viral envelope with the cellular plasma membrane. Cells infected by paramyxoviruses may also fuse with adjacent cells to form syncytia (multinucleated cells). Both virus-cell and cell-cell fusion processes are mediated by the viral fusion (F) protein, a trimeric type I integral membrane glycoprotein.

For members of the *Paramyxovirinae*, including SeV, fusion mediated by the F glycoprotein requires participation of the homologous attachment protein (glycoprotein [G], hemagglutinin [H], or hemagglutinin-neuraminidase [HN]). It has been hypothesized that conformational changes occurring in the attachment protein following binding to target cell receptors are transduced to the F protein to activate it for fusion "at the right time and in the right place" (26–28). Sialic acid-containing gangliosides act as the cellular receptor for the Sendai virus HN attachment protein (31, 32). While the specific cell surface receptor for pneumoviruses is unknown, RSV has been shown to interact with cell surface glycosaminoglycans (GAGs), in particular, with heparan sulfate (12, 13, 18, 20, 25, 33). Furthermore, the fusion protein of both human and bovine RSV (BRSV), as well as human metapneumovirus (MPV) is sufficient to mediate attachment and fusion in the absence of the G attachment protein (2, 16, 21, 43, 44, 47, 48, 52). Thus, the role of pneumovirus attachment proteins in triggering fusion is currently unclear. Since RSV F protein (F_{RSV}) also binds to GAGs, the interaction between F_{RSV} and heparan sulfate on the target cell surface may mediate virus attachment in the absence of the G protein (14, 20, 48).

The paramyxovirus F protein is synthesized as an inactive precursor (F_0), which is cleaved at a mono- or multibasic cleavage site by cellular proteases to produce a fusion-competent, disulfide-linked F_2-F_1 complex (for a review, see reference 28). Cleavage is an absolute requirement for fusion since the newly formed F_1 N terminus contains a hydrophobic fusion peptide that is inserted into the cell membrane during fusion (40). Furthermore, the nature of the cleavage motif is a major determinant of virus pathogenicity (23). Sendai virus F protein (F_{SeV}) is cleaved at a monobasic cleavage site (R116) and thus requires the addition of trypsin to fuse cells in culture (22, 42). In contrast, RSV F protein (F_{RSV}) possesses two conserved multibasic furin-dependent cleavage sites (site I, RARR109; site II, KKRKRR136), separated by a region of 27 amino acids (pep27). The presence of two cleavage sites in F_{RSV} is unique among paramyxoviruses although two cleavage sites have been observed in the severe acute respiratory syndrome (SARS) coronavirus spike protein (1). Interestingly, while cell-cell fusion directed by F_{RSV} does not depend on coexpression of an attachment protein, there is a requirement for proteolytic processing at both cleavage sites, accompanied by removal of the

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